(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 January 2001 (25.01.2001)

PCT

(10) International Publication Number
WO 01/05997 A2

(51) International Patent Classification[7]: C12P 21/00

(21) International Application Number: PCT/US00/19212

(22) International Filing Date: 14 July 2000 (14.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/354,959 16 July 1999 (16.07.1999) US

(71) Applicant *(for all designated States except US)*: MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: DEMAIN, Arnold, L. [US/US]; Apt. 251, 65 Grove Street, Wellesley, MA 0248 (US). FANG, Aigi [US/US]; 402 Highland Avenue, Apt. 6, Somerville, MA 02144 (US).

(74) Agent: MAH, Stanley, C.; Choate, Hall & Stewart, Exchange Place, 53 State Street, Boston, MA 02109 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

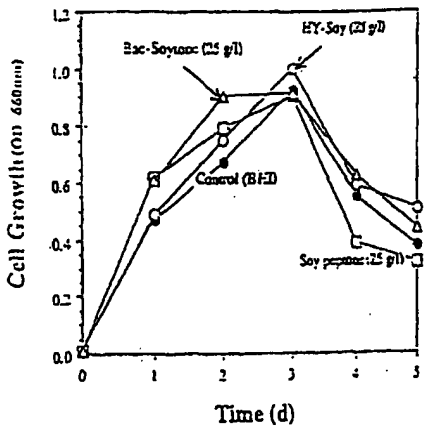
(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

[Continued on next page]

(54) Title: METHOD FOR PRODUCTION OF TETANUS TOXIN USING MEDIA SUBSTANTIALLY FREE OF ANIMAL PRODUCTS

The activity of soluble soy products as BHI replacements for cell growth.



(57) Abstract: The present invention provides a system for the growth of *C.tetani* and production of Tetanus Toxin for use in formulating Tetanus Toxoid preparations. The system includes growth media that contain significantly reduced levels of meat or dairy by-products using non-animal based products to replace the animal-derived products. Preferred media are substantially free of animal-derived products. Preferred media substantially free of animal-derived products comprise soy as a source of amino acids. The present invention also provides methods for production of Tetanus Toxin by culturing *C. tetani* in media substantially free of animal-derived products and comprising hydrolyzed soy, glucose and iron.

WO 01/05997 A2

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHOD FOR PRODUCTION OF TETANUS TOXIN USING MEDIA SUBSTANTIALLY FREE OF ANIMAL PRODUCTS

Priority Information

This PCT application claims priority to the US patent application U.S.S.N. 09/354,959 filed on July 16, 1999 by Dr. Arnold Demain and Dr. Aiqi Fang and is incorporated by reference herein in its entirety.

Background

Tetanus is a life-threatening disease caused by infection with *Clostridium tetani* (*C. tetani*), a ubiquitous anaerobic spore-forming soil microbe. *C. tetani* causes disease by releasing a peptide toxin, Tetanus Toxin, that enters the nerve cells of the infected host and blocks release of neurotransmitters at inhibitory synapses. This blockage produces unregulated excitation of certain host neurons, resulting in uncontrollable muscle contraction and paralysis, typically of facial and back muscles. As recently as 1989, 10-40% of *C. tetani* infections of non-immunized hosts resulted in death.

For the past 50 years, widespread immunization programs have been in effect to protect against the effects of *C. tetani* infection. Vaccines are prepared from Tetanus Toxin that has been inactivated, usually by exposure to formalin (Descomby, *Can. Roy. Soc. Biol.* 91:239, 1924; Plotkin et al., *Vaccines*, 2nd. Edition, W.B. Saunders, 1994). The inactivated Toxin is known as Tetanus Toxoid.

The *C. tetani* vaccination effort, although it has achieved significant success, has been hampered by certain complications associated with preparation of the Tetanus Toxoid. Because Tetanus Toxin is an extremely potent and dangerous compound (the estimated lethal dose for a human is only 2.5 ng/Kg), it is necessary to inactivate the Toxin very early in its purification process so that the risks of exposure to even partially purified active Toxin is minimized. As mentioned above, Tetanus Toxin is usually inactivated by exposure to formalin, which destroys the activity of the peptide toxin by generating inter-peptide cross-links and adducts. Unfortunately, formalin reactivity is not specific to Tetanus Toxin. A large number of other microbial and media proteins are also cross-linked by formalin. Because the Tetanus Toxin must

be treated with formalin at an early stage in its purification, the treatment produces a complex, heterogeneous composition containing not only formalin-inactivated Tetanus Toxin, but also formalin adducts of other peptides and proteins that were present in the formalin-treated mixture.

Tetanus Toxin is usually prepared from *C. tetani* cells that have been grown in media containing animal and dairy by-products (e.g., casein digests, meat extracts) as sources of the proteins, peptides, and amino acids that are required for growth or that stimulate growth. Tetanus Toxoid preparations generated from such cells necessarily contain some amount of formalin adducts of animal proteins. A possible consequence of this fact is that some Toxoid preparations could contain carry-over amounts of undesirable contaminants, such as the protein agent (prion) that causes Bovine Spongioform Encephalopathy (BSE), or antigenic peptides that stimulate undesired immune reactions (e.g., anaphylactic reactions) in immunized subjects.

The medium used to grow *C. tetani* for Tetanus Toxoid preparation has a variety of additional problems as well. The standard medium, known as "MM", was developed in 1954 by Mueller and Miller (*J. Bacteriol.* 67:271, 1954) and contains glucose, beef heart infusion (BHI), NZ-Case or NZ-Case TT (an enzymatic digest of casein), some amino acids and vitamins, uracil, and inorganic salts. The BHI component has proven to be particularly problematic, both because of the risk that it will contain undesirable products that may be carried over into the final Toxoid preparation (see, for example, Robb, *Proc. 4th Internat. Conf. Tetanus.*, Dankar, Senegal, pp. 735-43, 1975) and because of its variability from lot to lot.

The NZ Case component of MM medium has also caused problems due to its variability (see, for example, Stainer, *Proc. 4th Internat. Conf. Tetanus.*, Dakar, Senegal, pp. 745-54, 1975). It is now common practice for producers of Tetanus Toxoid preparations to screen numerous lots of NZ Case to identify a particular lot that results in high level Tetanus Toxin production. Once such a lot is identified, the Toxoid producer will typically purchase the entire lot.

Yet another problem with the MM medium typically used to culture *C. tetani* for Tetanus Toxoid preparation is that the medium has proven to be very sensitive to "cooking," in a manner that is independent of the requirement for sterilization. Apparently, extended cooking protocols can produce Maillard adducts and/or can degrade medium components such as proteins and

peptides, so that a less effective growth medium is produced when a particular batch of medium is imperfectly cooked.

Additionally, the process of *C. tetani* fermentation in MM medium generates H_2S, which is toxic to *C. tetani*, and may also generate some alcohols that can similarly be toxic. *C. tetani* growth in this medium, not to mention Toxin production, can be dramatically affected by the rate at which the head space in a fermenter is purged or gas exchanged, creating yet another point at which the extent and quality of Toxoid preparation can vary significantly from one batch to another.

There is a need for the development of an improved system for preparing Tetanus Toxoid that minimizes these risks and problems. Preferably, the system should allow for reproducible, high levels of Toxin production, and should minimize the dangers associated with formation of animal- or dairy-product adducts. There is a particular need for the development of a *C. tetani* culture medium and growth protocol that does not utilize meat or dairy by-products.

Summary of the Invention

The present invention provides a system for the growth of *C. tetani* and production of Tetanus Toxin for use in formulating Tetanus Toxoid preparations. The inventive system allows production of high levels of Tetanus Toxin and preferably utilizes at least one growth medium that contains significantly reduced levels of meat or dairy by-products as compared with MM medium; preferred media embodiments are substantially free of such components.

In one aspect, the present invention provides media that contain reduced levels of animal or dairy byproducts and are preferably substantially free of animal or dairy byproducts. For the purpose of the present invention, animal or dairy byproducts means any compound or collection of compounds that was produced in or by an animal cell, whether in a living organism or in vitro. Preferred non-animal sources of media ingredients such as proteins, amino acids, and nitrogen, include but are not limited to vegetables, microbes (such as yeast) and synthetic compounds.

In another aspect, the present invention provides methods of preparing Tetanus Toxin using at least one medium that is substantially free of animal or dairy byproducts. In one

embodiment, Tetanus Toxin is produced by culturing an organism of the genus *Clostridium* in a fermentation medium substantially free of animal products.

In another embodiment of the present invention, Tetanus Toxin is produced by culturing an organism of the genus *Clostridium* in a fermentation medium substantially free of animal products and containing vegetable-derived products.

In yet another embodiment, Tetanus Toxin is produced by culturing an organism of the genus *Clostridium* in a fermentation medium substantially free of animal products and containing soy-based products

In yet another preferred embodiment, Tetanus Toxin is produced by culturing *Clostridium tetani* in a fermentation medium substantially free of animal products and containing hydrolyzed soy as a substitute for animal-derived products. Preferably, growth in a fermentation medium proceeds until at least cell lysis occurs. The source of *C. tetani* used for inoculation of the fermentation medium may be obtained from a seed medium containing *C. tetani.* Preferably, *C. tetani* grown in a seed medium and used as an inoculant for a fermentation medium has not undergone cell lysis. The source of *C. tetani* used for inoculation of the seed medium may be obtained from a lyophilized culture. *C. tetani* may be lyophilized as a culture in animal milk or soy milk. Preferably *C. tetani* is lyophilized as a culture in soy milk.

In yet another preferred embodiment, Tetanus Toxin is produced by culturing *Clostridium tetani* in a fermentation medium substantially free of animal products and containing hydrolyzed soy as a substitute for animal-derived products. The fermentation medium further comprises iron, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, NaCl and glucose. The iron is any source of iron suitable for growth of *C. tetani*. Preferred sources of iron are iron powder, iron wire, iron foil, ferrous gluconate, ferrous citrate, and ferrous ammonium sulfate. In addition, culturing in a fermentation medium is preferably performed in the presence of nitrogen gas or a 90:10 mixture of nitrogen and hydrogen

The present invention also provides a composition comprising a medium substantially free of animal-derived products for culturing an organism and also comprising an organism of the genus *Clostridium.*

In one embodiment, the composition comprises a medium substantially free of animal-derived products while containing at least one product derived from a non-animal source, and also comprising an organism of the genus *Clostridium*.

In another embodiment, the composition comprises a medium substantially free of animal-derived products while containing at least one product derived from a vegetable, and also comprising an organism of the genus *Clostridium*.

In yet another embodiment, the composition comprises a medium substantially free of animal-derived products while containing at least one product derived from soybeans, and also comprising an organism of the genus *Clostridium*.

Description of the Drawings

Figure 1 presents a graphical representation of the requirement for BHI and NZ-CaseTT for *C. tetani* growth in control fermentation medium.

Figure 2 depicts the ability of various soluble soy products to replace BHI in fermentation media supporting growth of *C. tetani*.

Figure 3 depicts the ability of various insoluble soy products to replace BHI in fermentation media supporting growth of *C. tetani*.

Figure 4 depicts the ability of various soluble soy products to replace BHI in fermentation media supporting growth of *C. tetani*.

Figure 5 depicts the ability of various soluble soy products to replace BHI in fermentation media supporting growth of *C. tetani*.

Figure 6 shows a comparison of *C. tetani* cell growth supported by two different concentrations of BHI and two different concentrations of HY-Soy (Quest).

Figure 7 shows the ability of various soy products, present at 22.75 g/L, to replace BHI in fermentation media supporting growth of *C. tetani*.

Figure 8 shows the ability of various soy products, present at 22.75 g/L, to replace BHI for cell growth.

Figure 9 shows the effect on cell growth of NZ-CaseTT removal from various media employing BHI or soy peptones.

Figure 10 depicts the effect on cell growth of replacing NZ-CaseTT with yeast extract in media containing either BHI or a soy product.

Figure 11 is a graph showing the growth of *C. tetani* in the soy-based fermentation medium with inoculants originating from cultures of *C. tetani* lyophilized in animal milk or soy milk.

Figure 12 is a graph depicting growth of *C. tetani* in fermentation media containing yeast extracts or malt extracts as replacements for NZ-CaseTT

Detailed Description of Certain Preferred Embodiments of the Invention

The present invention provides a system for the growth of the microorganism *C. tetani,* and production of Tetanus Toxin by *C. tetani* for use in formulating Tetanus Toxoid preparations. The system includes growth media that contain significantly reduced levels of meat or dairy by-products; preferred media embodiments are substantially free of such components. Alternatively or additionally, the growth media lack one or more components found in MM medium. Preferably, the media lack one or both of BHI and NZ-Case.

The present invention encompasses the finding that animal-based products are not required as ingredients in media for the growth of *C. tetani*, and particularly that vegetable-based products can replace the animal-based products typically employed in such media for the growth of *C. tetani.* Currently, standard media that are commonly used for growth and production of products of fermentation by microorganisms contain ingredients derived from animals. One such growth medium is MM (Mueller and Miller. *J. Bacteriol.* 67:271, 1954). Animal components in MM include beef heart infusion (BHI), and NZ-CaseTT (hydrolyzed casein, a protein found in animal milk). Replacing the animal components of growth media with vegetable-based products reduces the potential for contamination by biological molecules such as proteins and viruses that exist in animals. By way of example and not limitation, these molecules include the protein agent that causes Bovine Spongioform Encephalopathy (commonly known as "Mad Cow's disease"), antigenic peptides that stimulate undesired immune reactions in immunized subjects (e.g., anaphylactic reactions), and virally-contaminated animal products.

In accordance with the present invention, preferred media for growth of *C. tetani* contain animal-derived ingredients comprising no more than 5-10% of the total weight of the media. More preferably, the growth media are substantially free of animal-derived products. Most preferably, all media and cultures used for the growth of *C. tetani* for the production of Tetanus Toxin are substantially or completely free of animal-derived products. These media include but are not limited to media for small and large scale fermentation of *C. tetani,* media for growth of cultures of *C. tetani* used to inoculate the fermentation media (*i.e.* seed media), and media used for long-term storage of cultures of *C. tetani* (*e.g.* stock cultures).

In certain preferred embodiments of the present invention, the media for the growth of *C. tetani* and production of Tetanus Toxin contain soy-based products that replace animal by-products. Preferably, these media include soybean by-products that are hydrolyzed and that are soluble in water. However, insoluble soy products can also be used in the present invention to replace animal products. Common animal by-products which can be substituted by soy products include, but are not limited to, beef heart infusion (BHI), hydrolyzed caseins, peptones, and dairy by-products such as animal milk. A non-limiting list of sources of hydrolyzed soy includes Hy-Soy (Quest International), Soy peptone (Gibco) Bac-soytone (Difco), AMISOY (Quest), NZ soy (Quest), NZ soy BL4, NZ soy BL7, SE50M (DMV International Nutritionals, Fraser, NY), SE50MK (DMV), and Marcor soy (soy peptones Tape AB, AC, SL, II and F; Marcor Development Co. Hackensack NJ, USA).

It is preferable that media containing soy-based products for the growth of *C. tetani* be similar to commonly used growth media containing animal derived products (*e.g* MM) except that substantially all animal-derived products are replaced with vegetable-derived products. Ingredients in MM such as Ca-pantothenate, uracil, thiamine, riboflavin, pyridoxine, and biotin are not essential for growth of *C. tetani* in media containing soy-based products, but can be included in media to enhance growth and toxin production. By way of example and not limitation, soy-based fermentation media can comprise a soy-based product, a source of carbon such as glucose, salts such as NaCl and KCl, phosphate-containing ingredients such as Na_2HPO_4, KH_2PO_4, divalent cations such as iron and magnesium, iron powder, and amino acids such as L-cysteine and L-tyrosine. Media used to grow cultures of *C. tetani* for inoculation (*i.e.* seed

medium) of the fermentation media preferably contain at least a soy-based product, a source of salt such as NaCl, and a carbon source such as glucose.

The present invention also provides a method for the growth of *C. tetani* that maximizes the production of Tetanus Toxin using media that are substantially free of animal-derived products. It is important to note that growth of *C. tetani* and production of Tetanus Toxin by *C. tetani* are not always directly correlated. Several factors including time of cell lysis and maximum levels of growth can influence the levels of toxin production. Growth of *C. tetani* for production of Tetanus Toxin proceeds by fermentation in media containing soy by-products that replace ingredients derived from animal by-products. The inoculant for the fermentation medium can be derived from a smaller scaled growth medium defined in the present application as a seed medium. Depending on the size and volume of the fermentation step, the number of successive growths in seed media to increase the biomass of the culture can vary. To grow a suitable amount of *C. tetani* for inoculating the fermentation medium, one step or multiple steps involving growth in a seed medium can be performed. For a method of growing *C. tetani* that is free of animal-derived products, it is preferable that growth of *C. tetani* originates from a culture stored in media lacking animal products and containing hydrolyzed soy. The stored culture, preferably lyophilized, is produced by growth in media containing proteins derived from soy and lacking animal by-products. It is recognized that growth of *C. tetani* in a fermentation medium may proceed by inoculation directly from a stored, lyophilized culture.

In a preferred embodiment of the present invention, growth of *C. tetani* proceeds in two phases—seed growth and fermentation—both of which are carried out in anaerobic environments, preferably with nitrogen gas or a mixture of nitrogen and hydrogen gas. The gas can either be bubbled through the medium during fermentation or passed through area above the liquid in the culture chamber (headspace). Preferably, the nitrogen gas or nitrogen/hydrogen mixture is passed through the headspace continuously. It is understood that production of Tetanus Toxin by *C. tetani* can also proceed in aerobic environments. However, maximal production of toxin occurs by fermentation. The seed growth phase is generally used to "scale-up" the quantity of the microorganism from a stored culture. The purpose of the seed growth phase(s) is to increase the quantity of the microorganism available as an inoculant for the

fermentation phase. In addition, the seed growth phase allows relatively dormant microbes in stored cultures to rejuvenate and grow into actively growing cultures. Furthermore, the volume and quantity of viable microorganisms used to inoculate the fermentation culture can be controlled more accurately from an actively growing culture than from a stored culture. Thus, growth of a seed culture for inoculation of the fermentation medium is preferred. In addition, any number of consecutive steps involving growth in seed media to scale-up the quantity of *C. tetani* for inoculation of the fermentation medium can be used. However, it is noted that growth of *C. tetani* in the fermentation phase can proceed directly from the stored culture by direct inoculation.

In the fermentation phase, a portion of a seed medium or all of a seed medium containing *C. tetani* from the seed growth is used to inoculate a fermentation medium. Preferably, approximately 2-4% of a seed medium having *C. tetani* from the seed growth phase is used to inoculate the fermentation medium. Fermentation is used to produce the maximum amount of microbe in a large-scale anaerobic environment (Ljungdahl and Wiegel. *Manual of industrial microbiology and biotechnology.* 1986. ed. Demain and Solomon. American Society for Microbiology, Washington, D.C. p. 84).

Tetanus Toxin may be isolated and purified using methods of protein purification well known to those of ordinary skill in the protein purification art (Coligan et al. "Current Protocols in Protein Science." Wiley & Sons; Ozutsumi et al. Appl. Environ. Microbiol. 49:939-943. 1985. Both citations are incorporated herein in their entirety).

Seed Medium

Preferably for production of Tetanus Toxin, cultures of *C. tetani* are grown in a seed medium for inoculation of the fermentation medium. The number of successive steps involving growth in a seed medium (i.e. inoculation of a second seed medium by a first seed medium) can vary depending on the scale of the production of Tetanus Toxin in the fermentation phase. However, as previously discussed, growth in the fermentation phase may proceed directly from inoculation from a stored culture. Animal-based seed media generally contain BHI, bacto-peptone, NaCl, and glucose for growth of *C. tetani*. As previously discussed, alternative seed

media may be prepared in accordance with the present invention in which animal-based components are substituted with non-animal-based components. For example but without limitation, soy-based products can substitute for BHI and bacto-peptone in the seed medium for growth of *C. tetani* and production of Tetanus Toxin. Preferably, the soy-based product is soluble in water and comprises hydrolyzed soy, although cultures of *C. tetani* can grow in media containing insoluble soy. However, levels of growth and subsequent toxin production are greater in media derived from soluble soy products.

Any source of soy-based products may be used in accordance with the present invention. Preferably, the soy is hydrolyzed soy. Sources of hydrolyzed soy are available from a variety of commercial vendors. These include but are not limited to Hy-Soy (Quest International), Soy peptone (Gibco) Bac-soytone (Difco), AMISOY (Quest), NZ soy (Quest), NZ soy BL4, NZ soy BL7, SE50M (DMV International Nutritionals, Fraser, NY), SE50MK (DMV), and Marcor soy (soy peptones Tape AB, AC, SL, II and F; Marcor Development Co. Hackensack NJ, USA). Most preferably, the source of hydrolyzed soy is Hy-Soy or SE50MK. Other sources of hydrolyzed soy have been tested and can be found in Tables 8-12.

Concentrations of Hy-Soy in the seed medium in accordance with the present invention range between 25-200 g/L. Preferably, the concentration of Hy-Soy in the seed medium ranges between 50-150 g/L. Most preferably the concentration of Hy-Soy in the seed medium is approximately 100 g/L.

In addition, the concentration of NaCl ranges between 0.1-2.0 g/L. Preferably the concentration of NaCl ranges between 0.2-1.0 g/L. Most preferably, the concentration of NaCl in the seed medium is approximately 0.5 g/L.

The concentration of glucose ranges between 0.1 g/L and 5.0 g/L. Preferably, the concentration of glucose ranges between 0.5-2.0 g/L. Most preferably, the concentration of glucose in the seed medium is approximately 1.0 g/L. It is also preferred but not necessary for the present invention that the glucose is sterilized by autoclaving together with the other components of the seed medium.

The preferred pH level of the seed medium prior to growth ranges between 7.5-8.5. Most preferably, the pH of the seed medium prior to growth of *C. tetani* is approximately 8.1.

Growth of *C. tetani* in the seed medium may proceed in one or more stages. Preferably, growth in the seed medium proceeds in two stages. In stage one, a culture of *C. tetani* is suspended in a quantity of seed medium and incubated at a temperature between 30-40 degrees C, preferably 34± 1° C for 24-48 hours in an anaerobic environment. In stage two, a portion or all of the stage one medium containing *C. tetani* is used to inoculate a stage two seed medium for further growth. After inoculation, the stage two medium is incubated also at a temperature between 30-40 degrees, preferably at 34± 1°C for approximately 1-4 days also in an anaerobic environment. Preferably, growth in the stage two seed medium proceeds for approximately 1-2 days. It is also preferable that growth in seed media in any stage does not result in cell lysis before inoculation of fermentation media with the final growth in seed medium. Additional growth in a third stage seed medium is optional.

Fermentation Medium

Standard fermentation media containing animal by-products for the growth of *C. tetani* are based on a recipe of Mueller and Miller (MM; *J. Bacteriol.* 67:271, 1954). The ingredients in MM media containing animal by-products include BHI and NZ-CaseTT. NZ-CaseTT is a commercially available source of peptides and amino acids which are derived from the enzymatic digestion of caseins, a group of proteins found in animal milk. The present invention encompasses the finding that non-animal based products may substituted for BHI and NZ-CaseTT in fermentation media. For example but without limitation, soy-based products can replace the animal-based components of MM media used for fermentation of *C. tetani*. Preferably, the soy-based products are water-soluble and derived from hydrolyzed soy, although as previously discussed, insoluble soy products can also be used to practice the present invention.

Any source of soy-based products may be used in accordance with the present invention. Preferably, the hydrolyzed soy is obtained from Quest International (Sheffield) under the tradename, Hy-Soy or from DMV International Nutritionals (Fraser, NY) under the tradename, SE50MK. Soluble soy products can be also obtained from a variety of sources including but not limited to Soy peptone (Gibco) Bac-soytone (Difco), AMISOY (Quest), NZ soy (Quest), NZ soy

BL4, NZ soy BL7, SE50MK (DMV International Nutritionals, Fraser, NY), Marcor soy (soy peptone Tape AB, AC, SK II, and F; Marcor Development Co., Hackensack, NJ USA).

In another preferred embodiment of the present invention, the medium used for fermentation of *C. tetani* is free of animal by-products and comprises hydrolyzed soy, glucose, NaCl, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, L-cysteine, L-tyrosine, and iron (preferably powdered iron or iron wire). As described for the seed medium, hydrolyzed soy can replace animal by-products in fermentation medium. These animal by-products include BHI and NZ-Case TT (enzymatically digested casein).

The concentration of Hy-Soy in the fermentation medium for production of Tetanus Toxin preferably ranges between approximately 10-100 g/L. Preferably, the concentration of Hy-Soy ranges between approximately 20-60 g/L. Most preferably, the concentration of Hy-Soy in the fermentation medium is approximately 35 g/L. For maximal production of Tetanus Toxin, particularly preferred concentrations of components in the fermentation medium are approximately 7.5 g/L, glucose; 5.0 g/L NaCl; 0.5 g/L Na_2HPO_4; 175 mg/L KH_2PO_4; 50 mg/L $MgSO_4 \cdot 7H_2O$; 125 mg/L L-cysteine; and 125 mg/L L-tyrosine. The amount of powdered iron used can range from 50 mg/L to 2000 mg/L. Preferably, the amount of powdered iron ranges between approximately 100 mg/L and 1000 mg/L. Most preferably, the amount of powdered iron used in fermentation media ranges between approximately 200 mg/L and 600 mg/L.

For optimal levels of toxin production, the initial pH (before autoclaving) of the soy-based fermentation media ranges preferably between approximately 5.5 to 7.1. Preferably the initial pH of the fermentation medium is between approximately 6.0 to 6.2. As described for the seed medium, the components of the fermentation medium, including glucose and iron, are sterilized preferably by autoclaving together for optimal production of toxin.

Preferably, a portion of the second stage seed medium used for growth of *C. tetani* is used to inoculate the fermentation medium. Fermentation occurs in an anaerobic chamber at approximately $34 \pm 1^\circ$ C for approximately 4 to 9 days. Growth is monitored by measuring the optical density (O.D.) of the medium. Fermentation preferably is stopped after cell lysis has proceeded for at least 48 hours as determined by growth measurement (optical density). As cells lyse, the O.D. of the medium will decrease.

Iron is an important ingredient in fermentation media for growth and toxin production. Any source of soluble and insoluble iron may be used in the present invention. Preferred sources of iron are powdered iron (e.g. reduced or non-reduced iron powder; J.T. Baker and Sigma-Aldrich), iron wire (e.g. Puratronic; Alfo Aesoar A Johnson Matthey Co.; and Sigma-Aldrich), iron foil, ferrous gluconate, ferric citrate and ferrous ammonium sulfate. Particularly preferred sources of iron are powdered iron and iron wire. When iron powder is used as the source of iron, it is preferred that the iron is autoclaved together with other ingredients of the fermentation medium which results in a higher level of toxin production compared to media with powdered iron autoclaved separately from the fermentation medium. Preferred iron wire has a diameter between approximately 0.05 mm and 2.0 mm. Particularly preferred iron wire has a diameter of 0.075 mm (e.g. Puratronic; 99.995% metal basis pure).

The use of activated charcoal in the fermentation medium also enhances production of Tetanus Toxin. Without being limited by theory, activated charcoal may absorb substances present in the medium and/or compounds produced by *C. tetani* which are inhibitory to toxin production.

In a preferred embodiment, *C. tetani* is cultivated by fermentation with continuous exposure to a 90% nitrogen/10% hydrogen mixture or 100% nitrogen. Optimal production of toxin resulted with continuous flow of nitrogen into the headspace. It is recognized that nitrogen gas or a mixture of nitrogen and hydrogen gas may be bubbled through the medium during fermentation. In addition, agitation (approximately 100 rpm) of the culture during fermentation resulted in increased toxin production. General methods of fermentation for *C. tetani* are well-known to those skilled in the art. (see De Luca et al. "Nitrogen-bas bubbling during the cultivation of *Clostridium tetani* produces a higher yield of tetanus toxin for the preparation of its toxoid." Microbiol Immunol. 41:161-13, 1997; and Nielsen, "Large-Scale production of tetanus toxoid." Appl. Microbiol. 15:453-4, 1967. Both references are incorporated by reference in their entirety.)

Preservation Medium

In a preferred embodiment of the present invention, cultures of *C. tetani* used for long-term storage of *C. tetani* and inoculation of the seed medium are grown and lyophilized in soy-milk prior to storage at 4 °C. Cultures of *C. tetani* in animal milk lyophilized for storage can also be used for the production of Tetanus Toxin. However, to maintain media that are substantially free of animal by-products throughout the production of Tetanus Toxin, it is preferred that the initial culture of *C. tetani* be preserved in soy milk and not animal milk.

Example 17 shows the results of experiments designed to examine the growth of *C. tetani* and the production of Tetanus Toxin in soy-based fermentation media using cultures of *C. tetani* lyophilized in animal-milk versus soy-milk as an inoculant. The results show that growth of *C. tetani* and production of Tetanus Toxin with lyophilized cultures of *C. tetani* in soy-milk is comparable to growth and production from cultures stored in animal milk.

Experimental Results

Soy Products Replacing Animal Products

Experiments were performed to test the ability of soy products to replace BHI for growth of *C. tetani* in fermentation media such as MM medium. The ingredients of the MM medium containing animal-based products are shown in Table 5. The results of these experiments are summarized in Examples 3-4, Tables 8-11 and Figures 2-8. These results indicate that soy-based products can replace BHI in media for the growth of *C. tetani*. Of the sources of soy tested, Hy-Soy and SE50MK were the best replacements for BHI for growth of *C. tetani* as measured by optical density. As indicated in Table 10, maximum growth of *C. tetani* was produced in a medium containing 34 g/L of Hy-Soy at day 3 or in a medium containing 34 g/L of SE50MK at day 3. Table 11a indicates that Marcor Soy peptone (Marcor Development Co. Hackensack, NJ) is also a suitable source of soy as a substitute for BHI and other animal-based products.

Additional experiments were performed to test concentrations of Hy-Soy suitable to replace animal-based products in media for growth of *C. tetani*. These experiments are summarized in Example 9 and Table 19. Concentrations of Hy-Soy ranging between

approximately 20 g/L to approximately 60 g/L in media were assayed. The results in Table 19 indicate that media with Hy-Soy and without BHI and NZ-CaseTT can support growth of *C. tetani*. Furthermore, the concentration of Hy-Soy resulting in the highest level of growth was approximately 58 g/L.

Since the levels of Tetanus Toxin production are not directly correlated with the levels of growth of *C. tetani*, the ability of soy-based products to replace animal-based products in media for production of Tetanus Toxin by *C. tetani* was additionally tested. Experiments summarized in Table 11 and Table 20 demonstrate that media containing hydrolyzed soy and substantially free of animal-based products are capable of supporting growth of *C. tetani* and production of Tetanus Toxin. An experiment summarized in Table 11 indicates that the highest levels of toxin produced resulted when media containing 23 g/L of Hy-Soy were used. Additional experiments summarized in Table 20 show that a concentration of Hy-Soy in media of approximately 35 g/L resulted in a high level of toxin production for the concentrations of Hy-Soy tested. It is noted that higher concentrations of Hy-Soy (57 g/L) resulted in higher levels of growth, but slightly lower levels of toxin production. Without limitation to theory, the lower production of toxin from higher levels of growth may be due to the lack of lysis at the end of the growth phase. Higher levels of Hy-Soy in media may be used to produce Tetanus Toxin. Therefore, Hy-Soy at concentrations ranging from approximately 10-100 g/L and preferably from approximately 20-60 g/L in fermentation media may be used as a substitute for animal-based products for production of Tetanus Toxin.

Experiments described in Example 6-8 were conducted to test the ability of non-animal derived products to replace NZ-CaseTT in fermentation media for growth of *C. tetani*. Table 13 in Example 6 shows that soy products can replace BHI and NZ-CaseTT for growth of *C. tetani*. Table 14 and Figure 10 show that a yeast extract can partially replace NZ-CaseTT in media for growth of *C. tetani*, but that corn steep, and Proflo, a yellow flour made from cottonseed (Traders Protein; Memphis TN) cannot be used to replace NZ-CaseTT in a fermentation medium. Table 15 and 16 summarize experiments examining the ability of yeast and malt extracts to enhance growth and toxin production by *C. tetani*. Table 15 indicates that growth of

C. tetani in media with Hy-Soy replacing BHI and NZ-CaseTT is enhanced by the addition of a yeast extract (see day 4 Hy-Soy only versus day 6 Hy-Soy plus yeast extract from Difco). However, yeast extracts did not improve the production of Tetanus Toxin in media containing Hy-Soy (Table 16).

Table 17 shows that yeast extracts except Hy-Yest 412 can partially replace NZ-CaseTT for growth. However, as indicated in Table 18 no yeast extract can replace NZ-CaseTT for toxin production. Malt extract had no ability to replace NZ-CaseTT. In addition, Hy-Soy without yeast extract is fully capable of substituting for animal by-products in media for toxin production. Therefore, it appears that the use of yeast extracts in growth media inhibits the production of toxin when used to substitute for NZ-CaseTT in the presence of Hy-Soy.

Seed media

Experiments summarized in Tables 23-24 were designed to examine the ability of Hy-Soy to replace BHI and NZ-CaseTT in seed media. Results in Tables 23-24 demonstrate that Hy-Soy or Hy-Soy plus Hy-Yest can replace BHI and bacto-peptone in seed growth. Preferably, the seed medium utilizes approximately 100 g/L of Hy-Soy as a nitrogen source replacing animal by-products. Table 24 summarizes experiments examining the growth of *C. tetani* in fermentation media containing Hy-Soy, Hy-Soy plus NZ-Case TT, or BHI plus NZ-Case TT. The three fermentation media listed were inoculated with *C. tetani* grown in the four different seed media described in Table 23 (A-D). The data in Table 23 and 24 indicate that growth of *C. tetani* in seed media and fermentation media with Hy-Soy replacing BHI and NZ-CaseTT is comparable to growth in media with BHI and NZ-CaseTT.

Furthermore, the data in Table 25 indicate that toxin production by *C. tetani* grown in seed medium and fermentation medium containing Hy-Soy as a replacement for BHI and NZ-CaseTT reached (or exceeded) levels attained in media containing BHI and NZ-CaseTT. For a comparison, see Fermentation nitrogen source: Hy-Soy only; row B and C; day 9 (Lf toxin=57.5) versus Fermentation nitrogen source NZ-CaseTT plus BHI row A-D (Lf toxin=42.5).

Additional experiments were performed to examine the effects of varying the concentration of Hy-Soy and Hy-Yest in seed media on growth and toxin production. Tables 26-27 summarize data on the growth of *C. tetani* in seed media with different concentrations of Hy-

Soy (B and C) or HySoy plus Hy Yest (D). Tables 26-27 demonstrate that Hy-Soy can replace BHI and bacto-peptone as the source of nitrogen in the seed medium and that Hy-Soy is a better source of nitrogen than [HySoy + Hy Yest] in seed medium for subsequent toxin production. Moreover, Tables 26-34 show that of the concentrations of Hy-Soy tested, 100 g/L of HySoy in a seed medium with subsequent inoculation of a fermentation medium results in the highest levels of Tetanus Toxin produced. Preferably, the concentration of Hy-Soy in the seed medium for optimal production of Tetanus Toxin is between approximately 25-200 g/L. More preferably, the concentration of Hy-Soy in the seed medium for optimal production of Tetanus Toxin is between approximately 50-150 g/L. Preferably, the first stage of growth in the seed medium proceeds for approximately 48 hours.

Experiments summarized in Example 12 (Tables 32-34) assay varying percentages of the second stage seed growth of *C. tetani* used to inoculate a fermentation medium. In addition, these experiments examined the effects of varying the period of growth for the second stage seed on the production of toxin in the fermentation step. The results show that using *C. tetani* grown in the second stage seed medium for 1-4 days is suitable for growth and toxin production. After 4 days, the cells appear to lyse. Preferably, the second stage seed medium containing *C. tetani* is incubated for growth for approximately 3 days. Use of 2-4% of the second stage seed medium containing *C. tetani* grown for 3 days as an inoculum for full scale fermentation appears to be optimal and preferred for Tetanus Toxin production.

Iron

Iron is an important ingredient in media for the production of Tetanus Toxin by *C. tetani*. Experiments described in Example 13 and 16 show that iron powder is an excellent source of iron for media used in accordance with the present invention. Other sources of iron suitable for toxin production include but are not limited to iron wire, iron foil, ferrous gluconate, ferrous citrate and ferrous ammonium sulfate.

Concentrations of iron powdered in media ranging from approximately 0 g/L to 1000 milligrams/L were assayed for the effects on growth and toxin production. The results are shown in Table 44. The results demonstrate that the lack of iron in media severely limited growth and

toxin production. The presence of iron powdered greatly enhanced growth and the production of toxin but that the differing concentrations did not greatly alter the levels of toxin production.

A separate experiment designed to test the affects of different iron powder concentrations (from 0-2.0 grams/L) autoclaved together with media or separately from the media demonstrated that 0.25 grams/L of iron powder was optimal for toxin production (Table 37). The results summarized in Tables 35-38 indicate that the concentration of powdered iron affected the levels of toxin production when the powdered iron is autoclaved with other components in the medium. The data (Table 37) show that the medium labeled MC1 containing 0.25 g of iron per L of the control medium when autoclaved together resulted in the highest levels of Tetanus Toxin production. This medium differs from control medium (labeled MC2) only by the amount of powdered iron (0.25 g vs. 0.50 g). Iron concentration did not appear to affect the levels of toxin production when the iron was autoclaved separately from the rest of the media.

The experiment in Example 16 demonstrates that 0.5 g/L of powdered iron in the fermentation medium combined with the use of approximately 2% of the second stage seed culture as an inoculum is preferred for the production of Tetanus Toxin. Toxin production is low in medium without an iron source. In addition, sources of iron such as ferrous sulfate, ferric citrate and ferric nitrate were not able to fully replace powdered iron for optimal production of toxin. However, growth of *C. tetani* and toxin production was observed when ferric citrate is used as the source of iron. Since previous experiments demonstrated that 0.25 g/L of powdered iron in fermentation media results in a high level of Tetanus Toxin produced, the amount of powdered iron in fermentation media for optimal production of Tetanus Toxin can range between approximately 0.25 g/L to approximately 0.50 g/L.

Experiments summarized in Table 46 show that ferrous gluconate can be used as a source of iron. Although levels of toxin production using media containing ferrous gluconate as the iron source were slightly lower than using media with iron powder, toxin production was achieved and was optimal at approximately 200 mg/L. Toxin production was observed for ferrous gluconate levels between approximately 200-800 mg/L.

Experiments summarized in Table 47 provide the unexpected result that iron wire can be used as a source of iron during growth of *C. tetani* for Tetanus Toxin production. Table 47

shows that iron wire with a diameter between approximately 0.075 millimeters and 1.2 millimeters, when used as the source of iron for growth of *C. tetani* in soy-based media, levels of toxin production are comparable to levels obtained when using iron powder. The highest levels of toxin production achieved for the five different iron wire amounts shown in Table 47 was observed for iron wire having a diameter of 0.075 provided at 1000 mg/L. Use of iron wire in media during fermentation of *C. tetani* will allow easy removable of iron after fermentation by simple extraction of the wire as compared to the relatively burdensome process of removing powdered iron.

Ferrous citrate is also an acceptable source of iron for growth of *C. tetani* and production of Tetanus Toxin. Interestingly, when activated charcoal is added to media containing ferrous citrate, toxin production is enhanced. Table 48 summarizes the results of an experiment on the use of activated charcoal in growth media.

Example 14 describes experiments designed to study the effect of different methods of media sterilization in the seed medium on the production of toxin by *C. tetani* in the fermentation medium. The results show that *C. tetani* grows faster in seed medium containing glucose that was autoclaved with the other components of the seed medium when compared to media containing filter-sterilized glucose or glucose autoclaved separately. Tetanus Toxin production was slightly higher in fermentation medium when glucose was autoclaved with the other fermentation medium components than when glucose was autoclaved separately or filtered separately. Thus, autoclaving glucose with the rest of the medium is somewhat beneficial for toxin production in fermentation medium.

Tables 41-42 in Example 15 show that the components of the Mueller and Miller medium not present in our Hy-Soy medium when added to medium containing Hy-Soy as the major source of nitrogen do not markedly improve growth or toxin production in the Hy-Soy fermentation medium. The best production was observed with Hy-Soy seed in combination with Hy-Soy fermentation medium.

Fermentation

In a preferred embodiment of the present invention, production of Tetanus Toxin by *C. tetani* using media containing soy and free of animal-based products is performed by culturing *C. tetani* in an anaerobic environment while passing gas (or a mixture of hydrogen and nitrogen, preferably 10% hydrogen:90% nitrogen) through the space in the fermentor above the liquid medium (headspace). Table 56 shows that toxin production was observed when either nitrogen gas or a 10:90 mixture of hydrogen:nitrogen gas was continuously passed into the headspace for most or all of the fermentation. For the experiment summarized on Table 56, optimal toxin production occurred in the fermentor supplied with 100% nitrogen during the first 6 days of fermentation. It is also preferred that the fermentation of *C. tetani* occur with gentle agitation (approximately 100 rpm) of the media during fermentation.

It is within the scope of the present invention for production of Tetanus Toxin to use any source of iron for fermentation of *C. tetani.* For large scale fermentation, preferred sources of iron are iron wire, iron powder, iron foil, ferrous gluconate, ferrous citrate and ferrous ammonium sulfate. More preferable source of iron are iron wire and iron powder. The use of activated charcoal is optional and may be preferred if levels of toxin production are not optimal.

The following examples are meant to illustrated the preferred method of practicing the present invention. However, it is known by one of ordinary skill in the art that variations, modifications and adaptations to the present invention can provide similar results. It is expressly understood that such variations, modifications, and adaptations are within the scope of the present invention.

EXAMPLES

Materials and Methods

Microorganism

A lyophilized culture of *C. tetani* (preparation 3-ABI-13; prepared with cow's milk), stored at 4 °C, was obtained from Wyeth-Lederle Vaccine and Pediatrics (Pearl River, NY). A lyophilized culture of *C. tetani* (preparation SM-409-0998-1; prepared with soy milk), stored at 4 °C, was obtained from Wyeth-Lederle Vaccine and Pediatrics (Pearl River, NY).

All experiments and media were prepared with doubly-distilled water.

Seeding

SEED MEDIUM COMPOSITION: Seed medium was prepared from the following components (per 100 ml of medium):

Table 1

NaCl	0.5 g
Bacto-peptone	1.0 g
Glucose	1.0 g
BHI	to 100.0 ml
pH (adjusted with 5 N NaOH)	8.1

SEED CULTURE: Lyophilized culture was suspended in 1 ml seed medium, split into two tubes each containing 10 ml seed medium, and incubated at 34° C for 24-48 hours. One ml of culture was then used to inoculate a 125 ml DeLong Bellco Culture Flask containing 40 ml of seed medium. The inoculated culture was incubated at 33±1° C for 24 hours in a Coy Anaerobic Chamber (Coy Laboratory Products Inc., Grass Lake, MI).

Fermentation

BASAL FERMENTATION MEDIUM COMPOSITION: Basal fermentation medium was prepared from the following components (per 1 L of medium):

Table 2

Glucose	7.5 g
NaCl	5.0 g
Na_2HPO_4	0.5 g
KH_2PO_4	0.175 g
$MgSO_4 \cdot 7H_2O$	0.05 g
Cysteine-HCl	0.125 g
Tyrosine-HCl	0.125 g
Powdered iron	0.5 g

CONTROL FERMENTATION MEDIUM COMPOSITION: Control fermentation medium was prepared from the following components (per 1 L of medium):

Table 3

BHI	250.0 ml[1]
NZ-CaseTT	15.0 g
Basal medium	to 1.0 L
pH	6.8

Basal medium was first prepared and adjusted to pH 6.8. BHI was then prepared, adjusted to pH 6.8 with 5 N NaOH, and added to basal medium. NZ-CaseTT was then prepared, added to basal

[1] Difco Manual (pg. 288) states that 500 g of fresh beef heart corresponds to 100 g dry weight. Wyeth-Lederle Vaccines & Pediatrics uses 455 g fresh beef heart to make 1 L of BHI, which should contain 91 g of dry weight. If this is correct, and BHI contains 91 g beef liver dry weight/L, then 250 ml BHI corresponds to 22.75 g dry weight of beef heart infusion.

medium + BHI, and dissolved by addition of HCl. pH was then adjusted to 6.8 with 5 N NaOH. Medium was distributed in 8 ml aliquots in 16 x 100 mm test tubes, and was autoclaved for 25 min at 121 °C.

TEST FERMENTATION MEDIUM COMPOSITION: Test fermentation media were prepared by substituting a test nitrogen source for the BHI present in the control fermentation medium. Test nitrogen sources included: Hy-Soy (Quest), AMI-Soy (Quest), NZ-Soy (Quest), NZ-Soy BL4 (Quest), NZ-Soy BL7 (Quest), Sheftone D (Sheffield), SE50M (DMV), SE50 (DMV), SE%)MK (DMV), Soy Peptone (Gibco), Bacto -Soyton (Difco), Nutrisoy 2207 (ADM), Bakes Nutrisoy (ADM) Nutrisoy flour, Soybean meal, Bacto-Yeast Extract (Difco) Yeast Extract (Gibco), Hy-Yest 412 (Quest), Hy-Yest 441 (Quest), Hy-Yest 444 (Quest), Hy-Yest (455 (Quest) Bacto-Malt Extract (Difco), Corn Steep, and Proflo (Traders).

Test media were prepared as described above for control medium except that BHI was not utilized and the relevant nitrogen source was first adjusted to pH 6.8 with 3 N HCl or 5 N NaOH. Media were distributed in 8 ml aliquots per 16 x 100 mm test tubes, and were autoclaved for 25 min at 121 °C.

CULTIVATION: A 40 µl aliquot of seed culture was used to inoculate each 8 ml control or test fermentation medium aliquot in an 8 ml 16 x 100 mm test tube. Cultures were then incubated at 33 ± 1 °C for 24 hours. Tubes were incubated in an anaerobic chamber to allow for growth. Each medium test was performed in triplicate (i.e., involved three independent inoculations of the same medium), and further included a non-inoculated control (used to blank the spectrophotometer). Growth (OD) was measured every 24 hours with a Turner Spectrophotometer (Model 330) at 660 nm. Cultivation was ceased after cell lysis had lasted for about 48 hours; toxin production was measured at that point.

Preparation of fermentation medium

COMPOSITION OF HY-SOY FERMENTATION MEDIUM: Experiments were performed with an Hy-Soy fermentation medium containing the following components per 1 L of medium:

Table 4

Hy-Soy	35.0 g
Glucose	7.5 g
NaCl	5.0 g
Na_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.05 g
KH_2PO_4	0.175 g
L-Cysteine	0.125 g
L-Tyrosine	0.125 g
Powdered iron	0.5 g
pH	6.8

COMPOSITION OF M&M FERMENTATION MEDIUM: M&M medium was prepared containing the following components per 1 liter of medium (see Mueller & Miller, *J. Bacteriol.* 67:271, 1954):

Table 5

NZ-CaseTT	22.5 g
BHI	50.0 ml
Glucose	11.0 g
NaCl	2.5 g
Na_2HPO_4	2.0 g
$MgSO_4 \cdot 7H_2O$	0.150 g
KH_2PO_4	0.150 g
L-Cysteine	0.250 g
L-Tyrosine	0.500 g
Powdered iron	0.5 g
Ca-pantothenate	1.0 mg
Uracil	2.5 mg
Thiamine	0.25 mg
Riboflavin	0.25 mg
Pyridoxine	0.25 mg
Biotin	2.5 μg
pH	7.0

PREPARATION OF FERMENTATION MEDIUM FOR POWDERED IRON AUTOCLAVE TEST: An incomplete fermentation medium was prepared that was lacking powdered iron, and its pH was adjusted to 6.8 with 5 N NaOH. Two sets of tubes were then prepared, one in which powdered iron was introduced into tubes, autoclaved, and then combined with 8 ml incomplete medium (per tube) that had been separately autoclaved; and one in which powdered iron was introduced into tubes, 8 ml of incomplete medium was added, and the mixture was subsequently autoclaved.

CULTIVATION: For control and test media, *C. tetani* cultivation was performed in fermentation media as described below.

Analysis of Tetanus Toxin production

CULTURE PROCESSING: Cultured cells were centrifuged, and the pH of the supernatant was determined.

MEASUREMENT OF TETANUS TOXIN PRODUCTION PARAMETERS: The levels of Tetanus Toxin in a given sample are measured by adding a standard antitoxin and measuring the elapsed time before flocculation. Both Kf (the time required for flocculation to occur, in minutes) and Lf (the limit of flocculation; equivalent to 1 international unit of standard antitoxin, as established by flocculation) were determined. 4 ml of fermentation broth were taken from each fermentation tube for a given culture, and were combined together so that 12 ml total were mixed in a 15 ml centrifuge tube. The tubes were centrifuged at 5000 rpm (3400 x g) for 30 min at 4 °C. 1 ml aliquots of supernatant were then added to tubes containing 0.1-0.6 ml of standard Tetanus antiserum, and the tubes were carefully shaken to mix their contents. The tubes were then placed in a water bath at 45 ± 1 °C and the initial time was recorded. Tubes were checked frequently, and the time at which flocculation began was recorded as Kf. The concentration of toxin in the tube in which flocculation was first initiated was designated Lf_{FF}. The concentration of toxin in the tube in which flocculation was initiated second was designated Lf_{F}.

An alternative procedure may be used to minimize that amount of antitoxin used. In this alternative procedure, 7.5 ml of fermentation broth (2.5 ml from each tube) was placed in a 15 ml centrifuge tube, mixed and centrifuged at 5000 rpm (3440 x g) for 30 min at 4°C. The supernatant was used for the Lf-determination of toxin. 18.8 µl, 37.5 µl, 56.3 µl, 75.0 µl, 93.8 µl, or 112.5 µl of standard tetanus antiserum was pipetted into a series of 6x50 mm borosilicate glass round-bottom tubes. To each tube was added 187.5 µl test supernatant. The tube contents were mixed by vortexing and placed in a water bath at 45±1° C. The time was noted and the tubes were examined frequently. The time at which flocculation first occurred in a tube was recorded and noted as Kf. The tube showing the first flocculation was designated as Lf_{FF} and the second tube as Lf_{F}.

Preferably, the fermentation of *C. tetani* in media free of animal products and inoculated from cultures also free of animal products with soy-base products replacing these animal products produces an Lf_{toxin} of approximately 50 or more. Minimally, Lf_{toxin} equals

approximately 10. Preferably the Lf_{toxin} is at least 20. Most preferably the Lf_{toxin} is greater than 50.

CALCULATION OF AMOUNT OF TETANUS TOXIN: The Lftoxin value is determined from the LfFF and LfF values as follows:

$Lf_{FF} > Lf_F$ $\quad Lf_{toxin} = Lf_{FF} - (Lf_{FF} - Lf_F)/4$

$Lf_{FF} < Lf_F$ $\quad Lf_{toxin} = Lf_{FF} + (Lf_F - Lf_{FF})/4.$

Example 1

Comparison of Media Containing Different Iron Sources

The purpose of this experiment was to compare the color, clarity, and pH of media containing powdered Fe, Fe^{++}, and Fe^{+++}. Four different media were prepared and analyzed as presented below:

Table 6

		No iron	Iron powder (Fe) 0.5 g/L	Ferrous sulfate (Fe++) 2.5 g/L	Ferric sulfate (Fe+++) 2.1 g/L
BEFORE AUTO-CLAVING	COLOR	tea	tea	green	orange
	CLARITY	clear	clear	clear	cloudy
	pH	6.8	6.8	6.8	6.8
AFTER AUTO-CLAVING	COLOR	tea	tea	gray	light tea
	CLARITY	clear	clear	cloudy	clear
	pH	6.6	6.7	6.0	5.2

The results presented above indicate that the iron powder is the best for experiments involving optical determination of cell growth.

Example 2

Analyzing BHI and NZ-CaseTT Requirements

The purpose of this experiment was to determine the need for BHI and NZ-CaseTT for cell growth by *C. tetani* in fermentation media. The control medium in this Example is described below.

Table 7

Beef heart infusion (BHI)	250 ml/l
NZ-CaseTT	15 g/l
Glucose	7.5 g/l
NaCl	5.0 g/l
Na_2HPO_4	0.5 g/l
KH_2PO_4	175 mg/l
$MgSO_4 \cdot 7H_2O$	0.05 g/l
L-cystine-HCl	125 mg/l
L-tyrosine-HCl	125 mg/l
Iron Powdered	0.5 g/l
pH	6.8

Media were tested as follows:

Table 7a

Part	Media	Growth (OD_{660})				
		1(d)	2(d)	3(d)	4(d)	5(d)
A	Control	0.38	0.61	0.81	0.70	0.31
	Without BHI	0.12	0.14	0.14	0.06	0.01
B	Control	0.43	0.62	0.85	0.56	0.31
	Without NZ-CaseTT	0.06	0.05	0.05	0.05	0.05

The results obtained, which are graphically represented in Figure 1, indicate that (i) it is possible to follow *C. tetani* growth by OD_{660}; and (ii) both BHI and NZ-CaseTT are required for good growth of *C. tetani*.

Example 3

Ability of Soy Products to Replace BHI for Growth in Fermentation Media

The purpose of this experiment was to evaluate the ability of various soy products to support *C. tetani* growth in fermentation media lacking BHI. Media were tested as follows:

Table 8

BHI Replacement	g/l	Growth (OD_{660})				
		1(d)	2(d)	3(d)	4(d)	5(d)
Control (BHI)	22.75	0.47	0.67	0.92	0.55	0.38
Hy-Soy (Sheffield)	6.25 g	0.39	0.63	0.76	0.33	0.21
	12.50 g	0.43	0.67	0.79	0.42	0.29
	25.00 g	0.49	0.75	1.00	0.59	0.51
Soy peptone (Gibco)	6.25 g	0.45	0.68	0.78	0.25	0.22
	12.50 g	0.51	0.77	0.90	0.30	0.24
	25.00 g	0.62	0.79	0.90	0.39	0.32
Bac-Soytone (Difco)	6.25 g	0.43	0.68	0.82	0.29	0.21
	12.50 g	0.51	0.77	0.83	0.39	0.26
	25.00 g	0.61	0.90	0.91	0.63	0.44
Hy-Soy T** (Sheffield)	6.25 g	0.42	0.60	0.66	0.35	0.26
	12.50 g	0.37	0.61	0.71	0.42	0.29
Nutrisoy flour**	6.25 g	0.32	0.51	0.49	0.39	0.22
	12.50 g	0.33	0.41	0.47	0.32	0.20
Soybean meal**	6.25 g	0.39	0.57	0.53	0.33	0.12
	12.50 g	0.41	0.62	0.45	0.26	0.13
Bake Nutrisoy** (ADM)	6.25 g	0.38	0.58	0.50	0.38	0.18

** Water-insoluble soy products

The results obtained, which are graphically represented in Figures 2 and 3, indicated that soluble soy preparations could replace BHI for growth of *C. tetani*. The best concentration appeared to be 12.5 or 25 g/L. Hy-Soy (Sheffield) gave the highest growth. Insoluble soy preparations were less effective.

Example 4

Ability of Additional Soy Products to Replace BHI for Growth in Fermentation Media

The purpose of this experiment was to test the ability of additional soy products to replace BHI in fermentation media supporting growth of *C. tetani.* Media were tested as follows:

Table 9

BHI Replacement	g/l	Growth (OD_{660})				
		1(d)	2(d)	3(d)	4(d)	5(d)
Control (BHI)	22.75 (250 ml)	0.38	0.61	0.81	0.70	0.31
Hy-Soy (Quest)	6.25 g 25.00 g	0.32 0.40	0.57 0.68	0.71 0.91	0.63 0.84	0.23 0.47
AMISOY (Quest)	6.25 g 25.00 g	0.40 0.47	0.56 0.56	0.59 0.53	0.44 0.20	0.24 0.04
NZ Soy (Quest)	6.25 g 25.00 g	0.31 0.48	0.57 0.74	0.75 0.91	0.52 0.40	0.30 0.27
NZ Soy BL4 (Quest)	6.25 g 25.00 g	0.28 0.16	0.60 0.47	0.71 0.73	0.61 0.66	0.47 0.51
NZ Soy BL7 (Quest)	6.25 g 25.00 g	0.32 0.38	0.58 0.59	0.69 0.69	0.54 0.56	0.33 0.29
SE50M (DMV)	6.25 g 25.00 g	0.34 0.36	0.58 0.38	0.69 0.69	0.59 0.65	0.29 0.27
SE50MK (DMV)	6.25 g 25.00 g	0.31 0.43	0.60 0.69	0.77 0.91	0.81 0.97	0.57 0.57
Soy peptone (Gibco)	6.25 g 25.00 g	0.31 0.47	0.63 0.59	0.77 0.83	0.75 0.45	0.34 0.30
Bac-Soytone (Difco)	6.25 g 25.00 g	0.30 0.43	0.63 0.79	0.79 0.88	0.47 0.42	0.21 0.40

The results obtained, which are graphically represented in Figures 4 and 5, indicate that a variety of different soy products are capable of supporting *C. tetani* growth in fermentation media in the absence of BHI. SE50MK at 25 g/L gave the best results. Quest Hy-Soy is most likely the same product as Sheffield Hy-Soy used in Example 3.

Example 5

Ability of Soy Products to Support Toxin Production in Fermentation Media

The purpose of this experiment was two-fold: (i) to confirm the observation, reported above in Examples 3 and 4, that soy products are able to replace BHI for *C. tetani* growth; and (ii) to test the ability of these soy products to support Tetanus Toxin production by *C. tetani*. Media were tested for their ability to support *C. tetani* growth as follows:

Table 10

BHI Replacement	g/l	Growth (OD_{660})*				
		1(d)	2(d)	3(d)	4(d)	5(D)
BHI (250 ml)	22.75	0.45	0.63	0.71	0.53	0.39
(375 ml)	34.13	0.52	0.77	0.97	0.50	0.38
Hy-Soy	22.75	0.45	0.77	0.94	0.54	0.37
(Quest)	34.13	0.50	0.83	1.02	0.69	0.63
NZ Soy	22.75	0.49	0.75	0.65	0.39	0.30
(Quest)	34.13	0.61	0.87	0.65	0.46	0.39
NZ Soy BL4	22.75	0.50	0.79	0.83	0.79	0.63
(Quest)	34.13	0.58	0.83	0.92	0.90	0.75
NZ Soy BL7	22.75	0.46	0.64	0.63	0.42	0.28
(Quest)	34.13	0.46	0.67	0.65	0.49	0.31
SE50M	22.75	0.47	0.59	0.80	0.35	0.26
(DMV)	34.13	0.55	0.60	0.83	0.49	0.42
SE50MK	22.75	0.42	0.77	0.93	0.89	0.46
(DMV)	34.13	0.53	0.82	1.10	0.88	0.69
Soy peptone	22.75	0.51	0.73	0.89	0.49	0.29
(Gibco)	34.13	0.56	0.73	0.92	0.48	0.41
Bac-Soytone	22.75	0.49	0.84	0.93	0.48	0.34
(Difco)	34.13	0.55	0.97	0.90	0.59	0.51

*Before fermentation, the OD_{660} was <0.01.

The results obtained, which are graphically represented in Figures 6, 7, and 8, indicate that Quest Hy-Soy, DMV SE50MK, and Quest NZ-Soy were effective soy products in terms of their ability to replace BHI for *C. tetani* growth. These products were also able to replace BHI at a higher level than levels analyzed in prior Examples.

Media were also tested for their ability to support toxin production, as follows:

Table 11

BHI Replacement	(g/l)	Lf_{FF}	Lf_{F}	Lf_{toxin}
BHI (250 ml)	22.75	30	20	27.5
(375 ml)	34.13	30	40	32.5
Hy-Soy	22.75	50	60	52.5
(Quest)	34.13	40	30	37.5
NZ Soy	22.75	20	10	17.5
(Quest)	34.13	30	20	27.5
NZ Soy BL4	22.75	--	--	--
(Quest)	34.13	--	--	--
NZ Soy BL7	22.75	--	--	--
(Quest)	34.13	--	--	--
SE50M	22.75	--	--	--
(DMV)	34.13	--	--	--
SE50MK	22.75	40	30	37.5
(DMV)	34.13	--	--	--
Soy peptone	22.75	--	--	--
(Gibco)	34.13	--	--	--
Bac-Soytone	22.75	--	--	--
(Difco)	34.13	--	--	--

Reaction done in a water bath at 46±1°C. (--) In this table and all subsequent tables, -- indicates the lack of flocculation in the toxin assay.

The results presented above, which represent analyses of toxin present in 5th day broth, reveal that the soy products (such as Quest Hy-Soy, DMV SE50MK, and Quest NZ-Soy) that were optimal for growth were also effective at replacing BHI for toxin production. The best soy product for toxin production was Quest Hy-Soy at 22.75 g/l. Interestingly, higher concentrations of this product produced better growth but did not improve toxin production. Similar results were obtained with SE50MK, for which a higher concentration generated increased growth but

did not increase toxin production. NZ-Soy, on the other hand, gave higher growth and higher toxin production at its higher concentration.

Table 11a.

Cell growth and toxin production with different types and concentrations of Marcor soy peptones.*

Marcor Soy peptone	Maximum Cell growth (OD 660 nm)			Maximum Lf_{toxin}		
amount	15 g/l	25 g/l	35 g/l	15 g/l	25 g/l	35 g/l
AB	0.41	0.88	1.00Δ	12.5	22.5	32.5
AC	0.67	0.84	0.99	0	12.5	17.5
SL	0.48	0.72	0.97	0	17.5	17.5
II	0.42Δ	0.72Δ	0.97Δ	0	22.5	37.5
F	0.29	0.62	0.91	12.5	22.5	32.5
Hy-Soy (control)			0.59			37.5
*One expt.; Δ=black color						

Table 11a demonstrates that Marcor Soy Peptones are suitable sources of soy in media for production of Tetanus Toxin.

We tested the pH of our various fermentation media as made up, before and after autoclaving, and before and after fermentation. We found, as shown below, that pH rose during fermentation, but its final value did not correlate with growth or production.

Table 12

BHI Replacement	(g/l)	pH of Medium *before* adjustment	pH of Medium *before* autoclaving	Broth	
				after autoclaving, before fermentation	after fermentation
BHI (250 ml)	22.75	6.2	6.8	6.5	7.1
(375 ml)	34.13	6.2	6.8	6.5	7.4
Hy-Soy (Quest)	22.75	7.1	6.8	6.6	7.5
	34.13	7.1	6.8	6.6	7.6
NZ Soy (Quest)	22.75	6.6	6.8	6.5	7.5
	34.13	6.6	6.8	6.4	7.6
NZ Soy BL4 (Quest)	22.75	4.0	6.8	6.4	6.4
	34.13	4.0	6.8	6.4	6.5
NZ Soy BL7 (Quest)	22.75	7.1	6.8	6.4	6.6
	34.13	7.1	6.8	6.4	6.6
SE50M (DMV)	22.75	7.4	6.8	6.5	7.3
	34.13	7.4	6.8	6.6	7.5
SE50MK (DMV)	22.75	7.3	6.8	6.6	7.2
	34.13	7.3	6.8	6.5	7.4
Soy peptone (Gibco)	22.75	7.0	6.8	6.5	7.2
	34.13	7.0	6.8	6.5	7.4
Bac-Soytone (Difco)	22.75	7.1	6.8	6.5	7.0
	34.13	7.1	6.8	6.5	7.3

Example 6

Ability of Soy Products to Replace NZ-CaseTT for Growth and Toxin Production in Fermentation Media

The purpose of this experiment was to determine whether NZ-CaseTT was required for cell growth and toxin production when BHI had been replaced with a soy product. The effect of NZ-CaseTT removal on cell growth is presented for a variety of different media (where BHI or soy product was used at a concentration of 22.75 g/L) below in Table 13, and also in Figure 9:

Table 13

BHI Replacement (22.75 g/l)	Growth (OD_{660})*				
	1 (d)	2 (d)	3 (d)	4 (d)	5 (d)
With NZ-CaseTT (15 g/l)					
Control (BHI)	0.43	0.62	0.85	0.56	0.31
HY-Soy	0.40	0.71	0.94	0.79	0.45
NZ-Soy	0.49	0.77	0.83	0.32	0.23
SE50MK	0.48	0.76	1.00	0.71	0.50
Without NZ-CaseTT					
Control (BHI)	0.06	0.05	0.05	0.05	0.05
HY-Soy	0.16	0.27	0.35	0.45	0.50
NZ-Soy	0.15	0.23	0.23	0.24	0.23
SE50MK	0.28	0.43	0.55	0.67	0.45

*Before fermentation the OD_{660} was <0.01.

These data confirm that soy products can effectively replace BHI in the presence of NZ-CaseTT, and also demonstrate that these products have some ability to replace NZ-CaseTT as

well. That is, NZ-CaseTT is required for growth in BHI-based media, but is not required for growth in soy-based media. Removal of NZ-CaseTT from soy-based media reduced growth (approximately 2-4 fold), but did not eliminate it. In this experiment, the best soy product for growth both in the presence and the absence of NZ-CaseTT was SE50MK.

Example 7

Ability of Non-soy, Non-animal Products to Replace NZ-CaseTT for Growth and Toxin Production in Fermentation Media

The purpose of this experiment was to test the ability of a variety of non-soy, non-animal products (Difco yeast extract, corn steep, or Proflo) to replace NZ-CaseTT for cell growth and/or for toxin production. BHI and soluble soy products were utilized at approximately 23 g/L; NZ-CaseTT and its replacements were utilized at 15 g/L. The results of the cell growth tests are presented below in Table 14, and also in Figure 10.

Table 14

	Growth (OD_{660})*				
BHI Replacement	1(d)	2(d)	3(d)	4(d)	5(d)
With NZ-CaseTT					
BHI (control)	0.43	0.62	0.85	0.56	0.31
Hy-Soy	0.40	0.71	0.94	0.79	0.45
NZ-Soy	0.49	0.77	0.83	0.32	0.23
SE50MK	0.48	0.76	1.00	0.71	0.50
With yeast extract replacing NZ-CaseTT					
BHI	0.28	0.39	0.47	0.58	0.57
Hy-Soy	0.32	0.44	0.50	0.61	0.67
NZ-Soy	0.64	0.65	0.69	0.67	0.49
SE50MK	0.40	0.49	0.42	0.45	0.54
With corn steep replacing NZ-CaseTT					
BHI	0.09	0.09	0.10	0.11	0.12
Hy-Soy	0.18	0.22	0.28	0.36	0.36
NZ-Soy	0.36	0.36	0.14	0.10	0.10
SE50MK	0.26	0.27	0.24	0.22	0.19
With Proflo replacing NZ-CaseTT					
BHI	0.02	0.01	0.02	0.02	0.02
Hy-Soy	0.05	0.10	0.05	0.03	0.03
NZ-Soy	0.12	0.09	<0.01	<0.01	<0.01
SE50MK	0.08	0.20	0.01	<0.01	<0.01

*Before fermentation the OD_{660} was <0.01.

As can be seen, yeast extract was able to partially replace NZ-CaseTT for growth; corn steep and Proflo were poor replacements.

Example 8

Ability of Yeast Extracts and Malt Extracts to Replace NZ-CaseTT for Growth and Toxin Production in Fermentation Media

The purposes of experiments summarized in Example 8 were to compare the effects of substituting various yeast extracts and malt extracts for BHI and NZ-CaseTT in media containing Hy-Soy for growth of *C. tetani* and production of Tetanus Toxin. Table 15 summarizes data on experiments substituting yeast extracts from two sources and a malt extract for BHI and NZ-CaseTT in media containing Hy-Soy for growth of *C. tetani.*

Table 15

		Growth (OD_{660})						
BHI Replacement	g/l	1(d)	2(d)	3(d)	4(d)	5(d)	6(d)	7(d)
With NZ-CaseTT								
BHI	22.75	0.46	0.65	0.80	0.73	0.33		
Hy-Soy	22.75	0.39	0.69	0.83	0.65	0.35		
Without BHI & NZ-CaseTT								
Hy-Soy	22.75	0.20	0.34	0.46	0.56	0.55	0.33	0.27
With Hy-Soy (22.75 g/l) & without BHI & NZ-CaseTT								
Yeast extract (Difco)	7.5	0.20	0.33	0.45	0.61	0.74	0.77	0.63
	15.0	0.29	0.42	0.49	0.61	0.71	0.75	0.59
	30.0	0.41	0.47	0.43	0.57	0.71	0.59	0.43
	45.0	0.57	0.48	0.58	0.69	0.49	0.47	0.42
Yeast extract (Gibco)	7.5	0.20	0.33	0.47	0.62	0.53	0.47	0.32
	15.0	0.25	0.40	0.50	0.64	0.55	0.43	0.35
	30.0	0.47	0.59	0.67	0.69	0.61	0.55	0.37
	45.0	0.66	0.73	0.81	0.48	0.39	0.45	0.34
Malt extract (Difco)	7.5	0.14	0.24	0.34	0.44	0.50	0.57	0.43
	15.0	0.13	0.20	0.28	0.37	0.42	0.50	0.47
	30.0	0.08	0.14	0.19	0.27	0.29	0.37	0.40
	45.0	0.07	0.10	0.12	0.15	0.19	0.23	0.28

Before fermentation, the OD_{660} was <0.01.

The above data show that again Difco yeast extract at 15 g/l partially replaced NZ-CaseTT for growth. Lower and higher concentrations were no better. Gibco yeast extract at 45 g/l was slightly better than Difco yeast extract; lower concentrations were slightly poorer. Difco malt extract had no ability to replace NZ-CaseTT and in fact was slightly inhibitory for growth.

Table 16 shows the effects of substituting yeast extracts and malt extract for BHI and NZ-CaseTT on Tetanus Toxin production by *C. tetani* in media for growth of *C. tetani.*

Table 16

BHI Replacement	(g/l)	Time (day)	Kf	Lf		
				Lf_F	Lf_{FF}	Lf_{toxin}
With NZ-CaseTT						
BHI	250 ml	5	70	30	20	27.5
HY-Soy	22.75	5	80	40	30	37.5
Without BHI & NZ-CaseTT						
HY-Soy	22.75	7	60	40	30	37.5
With HY-Soy and Without BHI & NZ-CaseTT						
Yeast extract (Difco)	7.5	8	120	10	20	12.5
	15.0	8	--	--	--	--
	30.0	7	--	--	--	--
	45.0	7	--	--	--	--
Yeast extract (Gibco)	7.5	7	--	--	--	--
	15.0	7	--	--	--	--
	30.0	7	--	--	--	--
	45.0	7	--	--	--	--
Malt extract (Difco)	7.5	7	--	--	--	--
	15.0	7	--	--	--	--
	30.0	7	--	--	--	--
	45.0	7	--	--	--	--

These data show that yeast extracts interfered with toxin formation even when the assay was performed on broth from the lytic phase of the growth cycle. Also, the data suggest that HY-

Soy can replace both BHI and NZ-CaseTT for toxin production. However, a longer fermentation cycle of 1 or 2 days may be necessary.

Table 17 summarizes data on experiments similar to experiments depicted in Table 16 designed to study the effects of additional yeast extracts on the growth of *C. tetani* when substituted for BHI and NZ-CaseTT in growth media for *C. tetani.*

Table 17

Growth (OD660)										
Media	g/l	1(d)	2 (d)	3 (d)	4 (d)	5 (d)	6 (d)	7 (d)	8 (d)	9 (d)
With NZ-CaseTT										
BHI	22.75	0.42	0.64	0.84	0.72	0.35	0.26	0.28	0.30	0.29
HY-Soy	22.75	0.39	0.65	0.94	0.85	0.69	0.47	0.38	0.29	0.31
Without BHI & NZ-CaseTT										
HY-Soy	22.75	0.17	0.34	0.49	0.56	0.58	0.38	0.31	0.27	0.26
With HY-Soy and Without BHI & NZ-CaseTT										
Hy-Yest 412 (Quest)	7.5	0.20	0.23	0.25	0.28	0.39	0.53	0.57	0.44	0.28
	15.0	0.41	0.52	0.48	0.38	0.39	0.39	0.35		
	30.0	0.54	0.58	0.33	0.27	0.23				
Hy-Yest 441 (Quest)	7.5	0.19	0.31	0.39	0.51	0.64	0.56	0.50	0.43	0.37
	15.0	0.27	0.38	0.44	0.52	0.56	0.56	0.45		
	30.0	0.55	0.55	0.49	0.50	0.44				
Hy-Yest 444 (Quest)	7.5	0.18	0.30	0.35	0.55	0.75	0.75	0.50	0.40	0.39
	15.0	0.28	0.37	0.40	0.58	0.66	0.59	0.46		
	30.0	0.56	0.60	0.60	0.50	0.38				
Hy-Yest 455 (Quest)	7.5	0.24	0.36	0.47	0.66	0.81	0.60	0.45	0.41	0.41
	0.30	0.47	0.59	0.79	0.77	0.63	0.57			
	0.46	0.59	0.71	0.71	0.57					

Before fermentation, the OD_{660} was <0.01.

The results are similar to results summarized in Table 16. The yeast extracts with the exception of HY-Yest 412 did partially replace NZ-CaseTT for growth. The optimal concentration for growth of *C. tetani* was the lowest concentration studied (7.5 g/l) for HY-Yest 441, HY-Yest 444 and HY-Yest 455.

Table 18 shows the effects of substituting yeast extracts for BHI and NZ-CaseTT on Tetanus Toxin production by *C. tetani* in growth media.

Table 18

BHI Replacement	(g/l)	Time (day)	Kf	Lf		
				Lf_{FF}	Lf_{F}	Lf_{toxin}
With NZ-CaseTT						
BHI	22.75	5	75	30	20	22.5
		7	60	30	40	32.5
		9	54	30	40	32.5
HY-Soy	22.75	5	90	30	20	27.5
		7	78	40	30	37.5
		9	65	40	30	37.5
Without BHI & NZ-CaseTT						
HY-Soy	22.75	7	82	40	30	37.5
	22.75	9	73	40	30	37.5
With HY-Soy and without BHI & NZ-CaseTT						
Hy-Yest 412 (Quest)	7.5	9	--	--	--	--
	15.0	7	--	--	--	--
	30.0	5	150	10	20	12.5
Hy-Yest 441 (Quest)	7.5	9	>260	20	10	17.5
	15.0	7	--	--	--	--
	30.0	5	--	--	--	--
Hy-Yest 455 (Quest)	7.5	9	>260	20	10	17.5
	15.0	7	--	--	--	--
	30.0	5	--	--	--	--

As in Table 17, HY-Soy could replace both BHI and NZ-CaseTT for toxin production. However, as also determined in Table 17, yeast extracts were inhibitory to toxin production. Therefore, the experiments outlined in Example 8 showed that Hy-Soy can replace both BHI and

NZ-CaseTT in media for growth and for production of Tetanus Toxin by *C. tetani.* Furthermore, addition of yeast extracts to this medium interfered with Tetanus Toxin production.

Example 9

The purpose of experiments summarized in Tables 19-22 was to determine the effects of increasing the concentration of Hy-Soy from the concentration (22.75 g/L) used in Examples 3-6 in media lacking BHI and NZ-CaseTT for growth and production of Tetanus Toxin by *C. tetani.*

Table 19

Growth (OD_{660})										
BHI Replacement	g/L	1(d)	2 (d)	3 (d)	4 (d)	5 (d)	6 (d)	7 (d)	8 (d)	9 (d)
Experiment 1										
With NZ-CaseTT										
BHI	22.75	0.46	0.65	0.80	0.73	0.33				
HY-Soy	22.75	0.39	0.69	0.83	0.65	0.35				
Without BHI & NZ-CaseTT										
HY-Soy	22.75 34.13 45.50 56.88	0.20 0.25 0.30 0.35	0.34 0.45 0.53 0.60	0.46 0.66 0.77 0.93	0.56 0.73 0.85 0.85	0.55 0.75 0.77 0.89	0.33 0.58 0.75 1.02	0.27 0.48 0.78 1.03		
Experiment 2										
With NZ-CaseTT										
BHI	22.75	0.42	0.64	0.84	0.72	0.35	0.26	0.28	0.30	0.29
HY-Soy	22.75	0.39	0.65	0.94	0.85	0.69	0.47	0.38	0.29	0.31
Without BHI & NZ-CaseTT										
HY-Soy	22.75 34.13	0.17 0.24	0.34 0.43	0.49 0.67	0.56 0.73	0.58 0.64	0.38 0.50	0.31 0.47	0.27 0.54	0.26 0.46

The initial OD_{660} was <0.01.

The above data covering 2 experiments show that concentrations of HY-Soy greater than 22.75 indeed replaced both BHI and NZ-CaseTT. The concentration of Hy-Soy resulting in maximal growth of *C. tetani* was 56.88 g/L.

Table 20 summarizes the data on the production of Tetanus Toxin by *C. tetani* in fermentation media containing varying concentrations of Hy-Soy with and without BHI and NZ-CaseTT.

Table 20

BHI Replacement	g/l	Time (d)	Kf	Lf		
				Lf_{FF}	Lf_{F}	Lf_{toxin}
Experiment 1 *With NZ-CaseTT*						
BHI	22.75	5	70	30	20	27.5
HY-Soy	22.75	5	80	40	30	37.5
Without BHI & NZ-CaseTT						
HY-Soy	22.75	7	60	40	30	37.5
	34.13	7	60	40	50	42.5
	45.50	7	>180	30	20	27.5
	56.88	7	>180	30	20	27.5
Experiment 2 *With NZ-CaseTT*						
BHI	22.75	5	75	30	20	22.5
		7	60	30	40	32.5
		9	54	30	40	32.5
HY-Soy	22.75	5	90	30	20	27.5
		7	78	40	30	37.5
		9	65	40	30	37.5
Without BHI & NZ-caseTT						
HY-Soy	22.75	7	82	40	30	37.5
		9	73	40	30	37.5
	34.13	7	62	50	40	47.5
		9	61	50	40	47.5

The above data confirm that HY-Soy at 22.75 g/l completely replaced both BHI and HY-CaseTT for toxin production. Unlike the effect on growth where 56.88 g/l HY-Soy was best, 34.13 g/l HY-Soy was best for toxin production. However this could be due to the fact that cells from 45.5 and 56.88 g/l HY-Soy had not lyzed by the end of the experiment.

Table 21

Summary of important results dealing with cell growth and Tetanus Toxin production in different media

Growth (OD660)									
Media	1 (d)	2 (d)	3 (d)	4 (d)	5 (d)	6 (d)	7 (d)	8 (d)	9 (d)
Control (BHI + NZ-CaseTT)									
	0.47 0.38 0.45 0.43 0.46 0.42	0.67 0.61 0.63 0.62 0.65 0.64	0.92 0.81 0.71 0.85 0.80 0.84	0.55 0.70 0.53 0.56 0.73 0.72	0.38 0.31 0.39 0.31 0.33 0.35	0.26	0.28	0.30	0.29
Ave	0.44	0.64	0.82	0.63	0.35	0.26	0.28	0.30	0.29
HY-Soy with NZ-CaseTT & without BHI									
	0.45 0.40 0.39 0.39	0.77 0.71 0.69 0.65	0.94 0.94 0.83 0.94	0.54 0.79 0.65 0.85	0.37 0.45 0.35 0.69	0.47	0.38	0.29	0.31
Ave	0.41	0.71	0.91	0.71	0.47	0.47	0.38	0.29	0.31
HY-Soy without BHI & NZ-CaseTT									
22.57 g/l	0.16	0.27	0.35	0.45	0.50				
	0.20	0.34	0.46	0.56	0.55	0.33	0.27		
	0.17	0.34	0.49	0.56	0.58	0.38	0.31	0.27	0.26
	0.18	0.32	0.43	0.52	0.54	0.36	0.29	0.27	0.26
Average	0.25	0.45	0.66	0.73	0.75	0.58	0.48		
34.13 g/l									
Average	0.24	0.43	0.67	0.73	0.64	0.50	0.47	0.54	0.46
	0.25	0.44	0.67	0.73	0.70	0.54	0.48	0.54	0.46

*Each set of data represents a different experiment and each data point is the average of 3 tubes

Table 22

Production of Tetanus Toxin

Media	Time (day)	Kf	Lf_{FF}	Lf_{F}	Lf_{toxin}
Control (BHI + NZ-CaseTT)					
	5	60	30	20	27.5
	5	60	30	20	27.5
	5	70	30	20	27.5
	5	75	30	30	22.5
	7	60	30	40	32.5
	9	54	30	40	32.5
HY-Soy with NZ-CaseTT &without BHI					
	5	50	50	60	52.5
	5	80	40	30	37.5
	5	80	40	30	37.5
	5	90	30	20	27.5
	7	78	40	30	37.5
	9	65	40	30	37.5
HY-Soy *without BHI & NZ-CaseTT*					
22.75 g/l	7	60	40	30	37.5
	9	65	40	30	37.5
	7	82	40	30	37.5
	9	73	40	30	37.5
34.13 g/l	7	60	40	50	42.5
	9	75	30	40	32,5
	7	62	50	40	47.5
	9	61	50	40	47.5

Example 10

The purpose of experiments summarized in Example 10 was to determine if Hy-Soy or [Hy-Soy + Hy-Yest] can replace BHI and Bacto-peptone in media for seed growth of *C. tetani*. In addition, experiments in Example 10 were designed to determine the optimum concentrations of components in seed media to produce the maximum levels of Tetanus Toxin by *C. tetani*.

As indicated below in Tables 23-25, growth of *C. tetani* in seed media and in fermentation media containing Hy-Soy only (lacking BHI and Bacto-peptone) reaches levels similar to those attained for growth in media containing BHI. For a comparison, see Table 24; Fermentation nitrogen source: Hy-Soy only; row B (100 g Hy-Soy in seed medium) versus Fermentation nitrogen source: BHI + NZ-CaseTT; rows A-D.

Table 23

Seed growth*

Nitrogen source of seed medium (per liter)	Growth (OD 660nm) Stage 1	Growth (OD 660nm) Stage 2
A 1000 mL BHI + 10 g Bacto-peptone	0.14	0.32
B 100 g Hy-Soy	0.07	0.52 (48 h)
C 50 Hy-Soy	0.07	0.22
D 50 g Hy-Soy + 50 g Hy-Yest	0.12	0.66

* Optical Density was measured after 24 hours unless indicated otherwise.

Table 24

Growth in fermentation media inoculated with seed cultures (A-D)

Seed medium	Growth (OD 660 nm)*								
	1 d	2 d	3 d	4 d	5 d	6 d	7 d	8 d	9 d
Fermentation nitrogen source: NZ-case TT (15 g/l) + BHI (250 mL/L)									
A	0.41	0.67	0.86	0.41	0.22	0.20	0.20	0.20	0.19
B	0.45	0.71	0.97	0.91	0.31	0.23	0.25	0.25	0.23
C	0.43	0.68	0.85	0.21	0.22	0.27	0.33	0.32	0.21
D	0.41	0.65	0.85	0.41	0.26	0.24	0.21	0.20	0.19
Fermentation nitrogen source: NZ-case TT (15 g/l)+ HY-Soy (22.75 g/l)									
A	0.39	0.69	0.91	0.73	0.46	0.35	0.31	0.31	0.28
B	0.45	0.75	1.00	0.87	0.52	0.39	0.33	0.33	0.33
C	0.40	0.67	0.64	0.32	0.28	0.27	0.27	0.28	0.25
D	0.42	0.72	0.96	0.74	0.45	0.39	0.37	0.37	0.35
Fermentation nitrogen source: HY-Soy (35 g/l) only.									
A	0.25	0.49	0.72	0.89	0.73	0.55	0.49	0.48	0.44
B	0.30	0.54	0.80	0.89	0.69	0.53	0.45	0.41	0.44
C	0.25	0.47	0.68	0.41	0.38	0.37	0.36	0.36	0.36
D	0.26	0.52	0.77	0.88	0.59	0.53	0.51	0.49	0.46

*Before fermentation, the OD 660nm was 0.

Table 25 indicates that toxin production by *C. tetani* grown in seed medium and fermentation medium that is free of BHI and NZ-CaseTT reached (or exceeded) levels attained in media containing BHI and NZ-CaseTT. For a comparison, see Fermentation nitrogen source: Hy-Soy; row B and C; day 9 (Lf toxin=57.5) versus Fermentation nitrogen source NZ-CaseTT + BHI row A-D (Lf toxin=47.5).

Table 25

Toxin production with different seed cultures:

Seed medium	Kf 5 d	Kf 7 d	Kf 9 d	Lftoxin 5 d	Lftoxin 7 d	Lftoxin 9 d
Fermentation nitrogen source: NZ-case TT 15 g/l + BHI 250 ml/l						
A	63	60	52	37.5	37.5	42.5
B	66	52	51	42.5	47.5	42.5
C	76	60	52	32.5	32.5	32.5
D	70	65	51	32.5	37.5	32.5
Fermentation nitrogen source: NZ-case TT 15 g/l+ HY-Soy 22.75 g/l						
A	83	70	56	32.5	42.5	37.5
B	117	60	60	27.5	37.5	37.5
C	92	97	81	27.5	27.5	22.5
D	73	59	48	32.5	37.5	37.5
Fermentation nitrogen source: HY-Soy 35 g/l						
A	--	56	44	--	52.5	57.5
B	--	51	49	--	52.5	57.5
C	65	57	51	42.5	47.5	57.5
D	--	57	43	--	52.5	57.5

Tables 23-25 show that Hy-Soy or [Hy-Soy + Hy-Yest] can replace both BHI and Bacto-peptone for seed growth. In addition, the seed culture of Hy-Soy or [Hy-Soy + Hy-Yest]

supported excellent cell growth and toxin production in different fermentation media including fermentation media free of BHI and NZ-CaseTT. The optimal concentrations of Hy-Soy in the seed and fermentation media were assayed in the following examples and range from approximately 50-150 g/L Hy-Soy in the seed medium and 15-50 g/L Hy-Soy in the fermentation medium for both growth and for Tetanus Toxin production.

Example 11

The purpose of experiments summarized in Example 11 was to determine the optimum concentrations of Hy-Soy or [Hy-Soy + Hy-Yest] for growth in the seed medium (Table 26). In addition, the effects of different Hy-Soy or [Hy-Soy + Hy-Yest] in the seed medium on growth (Table 27) and Tetanus Toxin production (Table 28) in the fermentation stage were tested.

Table 26

Seed growth

Nitrogen source of seed medium (per liter)	Growth (OD 660nm) Step -1 seed 24 h	Step -2 seed 24 h	48 h
A BHI 1000 ml +Bacto-peptone 10 g	0.12	0.33	0.30
B1 Hy-Soy 50 g	0.03	0.14	0.36
B2 Hy-Soy 62.5 g	0.02	0.01	0.32
B3 Hy-Soy 75 g	0.03	0.02	0.41
B4 Hy-Soy 87.5 g	0.03	0.09	0.51
B5 Hy-Soy 100 g	0.02	0.00	0.38
C1 Hy-Soy 50 g + Hy-Yest 10 g	0.04	0.25	0.36
C2 Hy-Soy 50 g + Hy-Yest 20 g	0.07	0.47	0.41
C3 Hy-Soy 50 g + Hy-Yest 30 g	0.02	0.53	0.41
C4 Hy-Soy 50 g + Hy-Yest 40 g	0.02	0.56	0.42
C5 Hy-Soy 50 g + Hy-Yest 50 g	0.02	.027	.064

Table 27

Growth in Hy-Soy fermentation medium inoculated with different seed cultures

Seed medium	Growth (OD 660 nm)*								
	1 d	2 d	3 d	4 d	5 d	6 d	7 d	8 d	9 d
A	0.06	0.26	0.41	0.65	0.63	0.41	0.36	0.31	0.26
B1	0.27	0.48	0.67	0.32	0.24	0.22	0.21	0.20	0.17
B2	0.27	0.44	0.57	0.73	0.70	0.57	0.38	0.32	0.29
B3	0.28	0.45	0.63	0.39	0.23	0.22	0.18	0.18	0.17
B4	0.30	0.47	0.12	0.14	0.13	0.14	0.14	0.14	0.14
B5	0.26	0.36	0.48	0.60	0.52	0.37	0.27	0.24	0.21
C1	0.24	0.40	0.59	0.67	0.45	0.31	0.26	0.22	0.18
C2	0.20	0.36	0.58	0.63	0.39	0.33	0.29	0.25	0.23
C3	0.24	0.41	0.61	0.57	0.37	0.31	0.28	0.25	0.23
C4	0.25	0.41	0.60	0.56	0.35	0.29	0.27	0.23	0.21
C5	0.15	0.30	0.44	0.59	0.49	0.36	0.31	0.27	0.24

*Before fermentation, the OD 660nm was 0.

Table 28
Toxin production with different seed cultures

Seed medium	Kf 5 d	Kf 7 d	Kf 9 d	Lftoxin 5 d	Lftoxin 7 d	Lftoxin 9 d
A	--	86	70	--	32.5	27.5
B1	69	61	60	37.5	42.5	42.5
B2	--	62	48	--	42.5	47.5
B3	--	60	60	--	47.5	42.5
B4	>180	>180	>180	22.5	22.5	22.5
B5	--	62	40	--	42.5	47.5
C1	--	99	92	--	22.5	17.5
C2	--	91	94	--	27.5	17.5
C3	--	94	92	--	37.5	27.5
C4	--	91	92	--	32.5	17.5
C5	--	93	94	--	27.5	17.5

The experiments summarized in Tables 26-28 confirm that Hy-Soy can replace BHI and Bacto-peptone as the nitrogen source in seed medium for growth of *C. tetani* and for production of Tetanus Toxin in the subsequent fermentation phase. Also, Hy-Soy as nitrogen source in the seed medium, as compared to Hy-Soy plus Hy-Yest, produced higher levels of Tetanus Toxin in the subsequent fermentation step. The concentrations of Hy-Soy in seed medium that produce the best levels of toxin range from approximately 62.5 g/L to 100 g/L.

Tables 29-30 summarize additional experiments designed to determine the optimum concentrations of Hy-Soy in the seed medium for the maximum production of Tetanus Toxin by *C. tetani* by fermentation. As shown in Table 30, 50 g, 75 g and 100 g of Hy-Soy in the seed medium all resulted in production of Tetanus Toxin by fermentation of *C. tetani* that is comparable or exceeds levels of Tetanus Toxin production in seed medium containing BHI and Bacto-peptone as a nitrogen source.

Table 29

Seed growth

Nitrogen source of seed medium (per liter)	Growth (OD 660nm) Step -1 seed 24 h	Step -2 seed 48 h	48 h
1000 ml BHI+10 g peptone	0.15	0.40	0.30
50 g Hy-Soy	0.035	0.10	0.29
75 g Hy-Soy	0.01	0.18	0.38
100 g Hy-Soy	0.03	0.25	0.51

Table 30

Growth in Hy-Soy fermentation media inoculated with different seed cultures:

Seed medium N source (per liter)	Growth (OD 660 nm)*						
	1 d	2 d	3 d	4 d	5 d	6 d	7 d
1000 ml BHI + 10 g peptone	0.19	0.37	0.58	0.79	0.69	0.45	0.41
50 g Hy-Soy	0.15	0.32	0.56	0.74	0.63	0.49	0.39
75 g Hy-Soy	0.19	0.35	0.60	0.77	0.68	0.49	0.45
100 g Hy-Soy	0.22	0.41	0.63	0.38	0.25	0.24	0.23

*Before fermentation, the OD 660nm was 0.

Table 31

Toxin production with different seed cultures (7 days)

Seed medium	Kf	Lftoxin
1000 ml BHI + 10 g peptone	150	32.5
50 g Hy-Soy	150	47.5
75 g Hy-Soy	155	37.5
100 g Hy-Soy	58	57.5

The data in Tables 26-31 indicate that a concentration of 100 g/L Hy-Soy in the seed medium resulted in the highest levels of toxin production in the subsequent fermentation step. In addition, the data indicate that seed step-1 of Hy-Soy seed medium produced greater growth after *48 hours than after 24 hours.*

Example 12

The three experiments summarized in Tables 32-34 were designed to determine the optimum concentration of the second stage seed growth containing *C. tetani* and the optimum length of time for the second stage seed growth that will produce that highest levels of Tetanus Toxin.

Table 32

Growth of second stage seed culture

Expt.	OD 660nm					
	1 d	2 d	3 d	4 d	5 d	6 d
A 1000 mL BHI + 10 g Bacto-peptone	0.38	0.60	0.87	0.95	0.41	0.34
B 100 g Hy-Soy	0.31	0.61	0.90	--	--	--
C 50 Hy-Soy	0.46	0.77	1.03	--	--	--

Table 33

Growth of *C. tetani* in culture inoculated with seed medium grown for different days and inoculated with different percentages of seed media

Expt./Age of 2nd seed	Growth (OD 660nm)						
	1 d	2 d	3 d	4 d	5 d	6 d	7 d
A/1 d	0.20	0.39	0.61	0.77	0.74	0.57	0.40
A/2 d	0.23	0.43	0.67	0.79	0.71	0.49	0.38
A/3 d	0.25	0.44	0.69	0.82	0.66	0.45	0.38
A/4 d	0.21	0.40	0.68	0.83	0.72	0.48	0.36
A/5 d	0.19	0.34	0.57	0.77	0.78	0.55	0.40
A/6 d	0.16	0.28	0.45	0.61	0.75	0.68	0.51
B/1 d	0.22	0.42	0.59	0.73	0.66	0.47	0.40
B/2 d	0.21	0.38	0.66	0.75	0.70	0.55	0.44
B/3 d	0.21	0.42	0.68	0.78	0.66	0.47	0.39
C/1 d	0.23	0.47	0.75	0.87	0.78	0.61	0.49
C/2 d	0.21	0.47	0.75	0.85	0.75	0.55	0.41
C/3 d	0.24	0.48	0.77	0.86	0.74	0.55	0.42
Expt./Inoculum conc (%) *	Growth (OD 660nm)						
	1 d	2 d	3 d	4 d	5 d	6 d	7 d
C/0.25	0.21	0.45	0.72	0.86	0.77	0.61	0.47
C/0.5	0.23	0.47	0.75	0.87	0.78	0.61	0.49
C/1.0	0.24	0.51	0.79	0.86	0.74	0.59	0.45
C/2.0	0.27	0.54	0.80	0.86	0.73	0.58	0.44
C/4.0	0.30	0.56	0.84	0.85	0.71	0.51	0.40

*The inoculum was a 24 h second stage seed culture.

Table 34

Toxin production (after 7 days of fermentation)

Expt./Age of seed (d)	Kf	Lftoxin
A/1 d	61	52.5
A/2 d	65	52.5
A/3 d	52	57.5
A/4 d	49	52.5
A/5 d	58	47.5
A/6 d	113	37.5
B/1 d	71	37.5
B/2 d	71	42.5
B/3 d	66	47.5
C/1 d	68	37.5
C/2 d	65	52.5
C/3 d	58	57.5
Expt./Inoculum (%)	Kf	Lftoxin
C/0.25	63	37.5
C/0.5	68	37.5
C/1.0	73	47.5
C/2.0	59	52.5
C/4.0	61	52.5

The above data show that (i) the age of the second stage seed culture of 1 to 4 days supports for growth and production; (ii) 5 and 6 day seed cultures were poor; at 5-6 days, the seed culture has begun to lyze. (iii) Growth of the second stage seed for 3 days appeared optimal for production. (iv) Use of 2% and 4% second stage seed culture as inoculum appeared optimal for toxin production.

Example 13

Experiments summarized in this Example were designed to examine the effects of different iron concentrations in fermentation medium for growth of *C. tetani*. In addition, the effects of sterilizing iron alone or with the medium were examined.

Table 35

Basal Fermentation medium

Components	Medium composition (g/L)
Glucose	7.5
Hy-Soy	35.0
L-Cysteine	0.125
L-Tyrosine	0.125
NaCl	5.0
Na_2HPO_4	0.5
$MgSO_4$	0.05
KH_2PO_4	0.175

Variations Fermentation Media

Iron autoclaved separately	C1	C2	C3	C4
Iron autoclaved with medium	MC1	MC2	MC3	MC4
Concentration of powdered iron (g/L)	0.25	0.5	1.0	2.0

Initial pH = 6.8

Growth in fermentation media:

Table 36

Medium	Growth (OD 660nm)*								
	1 d	2 d	3 d	4 d	5 d	6 d	7 d	8 d	9 d
C1	0.21	0.41	0.55	0.70	0.63	0.49	0.39	0.32	0.29
C2 (control)	0.22	0.42	0.59	0.73	0.66	0.47	0.40	0.35	0.31
C3	0.20	0.45	0.66	0.79	0.68	0.50	0.45	0.39	0.35
C4	0.19	0.39	0.64	0.79	0.67	0.50	0.45	0.39	0.36
MC1	0.49	0.72	0.96	1.00	0.72	0.48	0.33	0.31	0.38
MC2 (control)	0.77	0.90	1.15	1.20	0.86	0.67	0.46	0.46	0.42
MC3	0.92	1.30	1.20	1.10	0.94	0.79	0.69	0.59	0.56
MC4	1.30	1.45	1.40	1.08	0.66	0.45	0.35	0.30	0.32

*Before fermentation, the OD 660nm was 0.

Table 37
Toxin production

Medium	Kf		Lftoxin	
	7d	9d	7d	9d
C1	87	79	32.5	47.5
C2 (control)	78	68	37.5	47.5
C3	72	70	42.5	47.5
C4	66	71	42.5	47.5
MC1	54	40	52.5	67.5
MC2 (control)	76	48	32.5	47.5
MC3	85	68	27.5	37.5
MC4	104	83	17.5	32.5

The data summarized in Tables 35-37 show that MC1 medium containing 0.25 g of iron per L of the control medium autoclaved together with the medium resulted in the highest levels of Tetanus Toxin production. This medium differed from control medium (MC2) only by the amount of powdered iron. The control MC2 medium contained twice as much powdered iron as MC1. For both media, the iron was autoclaved together with the other components. Thus, the concentration of powdered iron was a major factor influencing toxin production when the powdered iron was autoclaved with other components in the medium. Medium containing 0.25 g/L of powdered iron produced the highest levels of toxin, and medium containing 2 g/L of powdered iron produced the lowest levels. Iron concentration did not affect the levels of toxin production when the iron was autoclaved separately from the rest of the media.

Example 14

Experiments described in this example were designed to study the effect of different methods of media sterilization on toxin production. More specifically, the methods for sterilization of glucose in seed and fermentation media containing Hy-Soy were examined.

The three method of sterilization were as follows:

A. All components autoclaved together (standard procedure).

B. Glucose autoclaved separately.

C. Glucose sterilized by filtration with <0.2 μm filter.

Table 38
Seed growth:

Sterilization of glucose for seed	Seed growth (OD 660nm)*						
	Step-1	Step-2					
	2 d	1d	2 d	3 d	4 d	5 d	6 d
A	0.24	0.31	0.60	0.85	0.88	0.75	0.71
B	0.11	0.06	0.55	0.91	1.08	0.89	0.68
C	0.10	<0.01	0.45	0.63	0.85	0.98	0.91

Table 39

Effects of different methods of sterilization of seed media on growth in fermentation medium

Sterilization of glucose for fermentation medium	Growth (OD 660nm)*						
	1 d	2 d	3 d	4 d	5 d	6 d	7 d
All components of seed medium autoclaved together							
A	0.22	0.41	0.57	0.73	0.44	0.27	0.24
B	0.32	0.50	0.73	0.77	0.41	0.32	0.29
C	0.35	0.53	0.72	0.81	0.43	0.33	0.29
Glucose autoclaved separately in seed medium							
A	0.24	0.43	0.63	0.68	0.31	0.24	0.21
B	0.35	0.54	0.78	0.75	0.38	0.32	0.25
C	0.33	0.50	0.76	0.73	0.37	0.30	0.25
Glucose sterilized by filtration with <0.2 μm filter for seed medium							
A	0.21	0.38	0.60	0.74	0.62	0.40	0.34
B	0.36	0.49	0.71	0.84	0.63	0.41	0.31
C	0.34	0.48	0.69	0.85	0.63	0.42	0.35

*Before fermentation, the OD 660nm was 0.

Table 40

Effects on different methods of sterilization of seed media on toxin production (7 days)

Sterilization of glucose for fermentation medium	Kf	Lftoxin
All components of seed medium autoclaved together		
A	61	47.5
B	79	40.0
C	77	42.5
Glucose autoclaved separately in seed medium		
A	54	47.5
B	71	42.5
C	72	42.5
Glucose sterilized by filtration with <0.2 μm filter for seed medium		
A	72	47.5
B	96	42.5
C	93	42.5

The results shown in Tables 38-40 demonstrated that *Clostridium tetani* grew faster in seed medium when glucose was autoclaved with the other components of the seed medium as compared to media containing glucose that was autoclaved separately or sterile-filtered separately. Also, Tetanus Toxin production was slightly higher in fermentation medium when glucose was autoclaved with the other fermentation medium components than when glucose was

autoclaved separately or filtered separately. Thus, autoclaving glucose with the rest of the medium is beneficial for seed growth and toxin production in fermentation medium.

Example 15

Experiments in Example 15 were designed to determine the effects on growth and toxin production of adding growth factors of the Mueller and Miller (MM) medium to the fermentation medium containing Hy-Soy as the source of nitrogen. These growth factors include Ca-pantothenate, uracil, thiamine, riboflavin, pyridoxine, and biotin.

Table 41

Growth in fermentation media:

Fermentation medium	Growth (OD 660 nm)*						
	1 d	2 d	3 d	4 d	5 d	6 d	7 d
Seed medium: 1000 ml BHI + 10 g/l Bacto-peptone							
Hy-Soy	0.19	0.37	0.58	0.79	0.69	0.45	0.41
Hy-Soy + growth factors**	0.22	0.38	0.58	0.76	0.78	0.54	0.50
Mueller & Miller	0.44	0.70	0.76	0.80	0.44	0.32	0.30
Seed medium: 100 g/l Hy-Soy							
Hy-Soy	0.22	0.41	0.63	0.38	0.25	0.24	0.23
Hy-Soy + growth factors	0.24	0.43	0.61	0.73	0.32	0.29	0.27
Mueller & Miller	0.47	0.67	0.55	0.19	0.19	0.20	0.21

*Before fermentation, the OD 660nm was 0.

**Growth factors are Ca-pantothenate, 1 mg/l; uracil, 2.5 mg/l; thiamine, 0.25 mg/l; riboflavin, 0.25 mg/l; pyridoxine, 0.25 mg/l; biotin, 2.5 µg/l

Table 42

Toxin production

Fermentation medium	Kf			Lftoxin		
	5 d	6 d	7 d	5 d	6 d	7 d
Seed medium: 1000 ml BHI + 10 g/l Bacto-peptone						
Hy-Soy	--	>180	150	--	27.5	32.5
Hy-Soy + growth factors	--		>180	--	--	17.5
Mueller & Miller	--	54	60	--	17.5	17.5
Seed medium: 100 g/l Hy-Soy						
Hy-Soy	80	70	58	37.5	47.5	57.5
Hy-Soy + growth factors	--	76	59	--	47.5	57.5
Mueller & Miller	65	68	70	32.5	32.5	32.5

The results of experiments summarized in Table 41-42 demonstrate that growth factors of the Mueller and Miller medium added to medium containing Hy-Soy as the sole source of nitrogen may have a marginal effect on growth but did not improve toxin production in the Hy-Soy fermentation medium. The best production was observed with Hy-Soy seed in combination with Hy-Soy fermentation medium.

Example 16

Sources of iron.

Experiments in Example 19 were designed to examine the effect of various iron sources on the production of Tetanus Toxin. The results in Table 47 show that sources of iron such as ferrous sulfate, ferrous gluconate, ferric citrate and ferric nitrate were not able to fully replace powdered iron for production of toxin, although some toxin was produced with ferric citrate.

Seed medium (%):

Hy-Soy	10.0
NaCl	0.5
Glucose	1.0
pH	8.1

Fermentation medium:

Basal medium without Fe:

Hy-Soy	35.0 g/l
Glucose	7.5 g/l
NaCl	5.0 g/l
Na_2HPO_4	0.5 g/l
KH_2PO_4	175 mg/l
$MgSO_4$-$7H_2O$	50 mg/l
L-cysteine	125 mg/l
L-tyrosine	125 mg/l

Table 43

Iron Components	Grams/Liter			
Powdered iron	0.0625	0.125	0.25	0.50
Ferrous sulfate ($FeSO_4$-$7H_2O$)	0.625	1.25	2.50	
Ferric citrate ($FeC_6H_5O_7$)	0.55	1.10	2.20	
Ferric nitrate ($Fe(NO_3)_3 \cdot 9H_2O$)	0.90	1.80	3.60	

pH 6.8

Method:

As previously described, all media were prepared with double-distilled water. Iron-containing compounds listed in Table 43 were added to the fermentation medium lacking iron (basal medium). The final concentrations of ingredients in media are provided as grams/liter. The pH of the solutions were adjusted to 6.8 with 5 N NaOH. For purposes of sterilization, iron-containing compounds were autoclaved with the other ingredients of the media. Two stages of seed culture were used. The length of incubation for both seed cultures was approximately 48 hours. 2% of the second stage seed culture was used as an inoculum for the fermentation media.

Table 44 summarizes experiments assaying different amounts of iron powder in media for growth and toxin production by *C. tetani*. The presence of iron powdered greatly enhanced the levels of toxin production. Amounts of iron powder ranging from approximately 60 mg/L to 1000 mg/L were suitable for growth and toxin production.

Table 45 indicates the levels of toxin production after 7 days and 9 days of fermentation for different sources of iron.

Table 44

Toxin production with different concentrations of powdered iron.*

Reduced iron Power (mg/l)	Maximum cell growth (OD 660 nm)	Maximum Lf_{toxin}
0	0.30	8.3
62.5	0.59	52.5
125	0.60	52.5
250	0.63	47.5
500	0.65	57.5
1000	0.69	42.5
*Six expts.		

Table 45

Toxin production (on 7th day) with different concentrations of Fe components:

Ferrous Sulfate (g/l)	K_f	$L_f$toxin
0.625	>180	12.5
1.25	>180	12.5
2.50	>180	<7.5
Ferric citrate (g/l)		
0.55	160	22.5
1.10	165	17.5
2.20	165	17.5
Ferric nitrate (g/l)		
0.90	170	17.5
1.80	>180	<7.5
3.60	>180	<7.5

Table 46

Effect of ferrous gluconate ($C_{12}H_{22}FeO_{14}$)*.

Ferrous gluconate (mg/l)	Fe (mg/l)	Maximum cell growth (OD 660 nm)	Maximum Lf_{toxin}
4300	499	ND**	0
2150	249	ND	0
1075	125	ND	0
800	93	0.43	32.5
400	46	0.39	37.5
200	23	0.38	42.5
0	0	0.30	8.3
Reduced iron powder (Control)	500	0.71	64.6

*Two expts.
**ND=-not done

Table 46 summarizes the results of two experiments showing that ferrous gluconate is a suitable source of soluble iron for growth of *C. tetani* and Tetanus Toxin production. The optimum concentration ranged from approximately 200-800 mg/L with higher concentrations inhibiting toxin formation.

Table 47

Effect of replacing reduced iron powder with iron wire on growth and toxin formation.*

Iron source (mg/l)	Maximum cell growth (OD 660 nm)	Maximum Lf_{toxin}
None	0.26	6.7
Reduced iron powder (500) (Control)	0.65	62.5
0.075 mm diam. iron wire (500)	0.48	50.8
0.075 mm diam. iron wire (1000)	0.47	55.0
0.075 mm diam. iron wire (2000)	0.48	52.5
1.2 mm diam. iron wire (1000)	0.37	42.5
1.2 mm diam. iron wire (2000)	0.45	42.5
32 gauge galvanized iron wire (1000)	0.23	7.5
32 gauge galvanized iron wire (2000)	0.22	17.5
*Three expts.		

Table 47 summarizes results of experiments showing that iron wire is a good source of iron in media for growth of *C. tetani* and Tetanus Toxin production.. For wire with a diameter of 0.075 mm toxin production was comparable to production in media using iron powder. Thicker wire (1.2 mm) was also suitable as a source of iron. The results summarized in Table 47 show that iron wire is a suitable source of iron in media. Galvanized iron wire was not effective in these experiments.

Table 48

Effect of activated charcoal added to ferrous citrate on growth and toxin production.*

Ferric citrate (mg/l)	20-40 mesh Activated charcoal (mg/L)	Maximum cell growth (OD 660 nm)	Maximum Lf_{toxin}
140	0	0.52	42.5
550	0	0.46	42.5
140	250	0.54	57.5
140	500	0.51	57.5
550	500	0.48	57.5
Reduced iron powder (550 mg/1) (Control)		0.72	67.5
*Two expts.			

Table 48 shows that activated charcoal in media did not affect growth of *C. tetani* but enhanced toxin formation in the presence of ferric citrate, which is a suitable source of soluble iron. Although the combination of ferric citrate and activated charcoal did not fully replace reduced iron powder in media for toxin production, the yield with activated charcoal and ferric citrate was 85-94% of the yield for toxin production with reduced iron powder as the source of iron. The level of toxin production with reduced iron powder from nine experiments averaged 61.4 units. Without limitation to theory, the data suggest that reduced iron powder has two functions. One is to provide iron and the other is to absorb toxic material in the medium or toxic material produced by the cell.

Example 17

Storage of Cultures in Soy Milk

The purpose of experiments described in Example 17 was to determine the effects of using *C. tetani* cultures that were lyophilized and stored in soy milk at 4° C.

Microorganisms:

Clostridium tetani (preparation 3-ABI-13; lyophilized culture of animal milk, stored at 4° C).

Clostridium tetani (preparation SM-409-0998-1; lyophilized culture of soy-milk, stored at 4° C).

Seed medium (%):

Hy-Soy	10.0
NaCl	0.5
Glucose	1.0
pH	8.1

Fermentation medium:

Hy-Soy	35.0 g/l
Glucose	7.5 g/l
NaCl	5.0 g/l
Na_2HPO_4	0.5 g/l
KH_2PO_4	175 mg/l
$MgSO_4.7H_2O$	50 mg/l
L-Cysteine	125 mg/l
L-Tyrosine	125 mg/l
Powdered iron	0.5 g/l
pH	5.6-7.4

Methods:

1. Stage 1 and 2 seed cultures were both incubated for approximately 48 hours. The inoculum for the fermentation phase was 2% of second stage seed culture. Growth in seed media and fermentation were performed as previously described.

Results:

Table 49

Seed culture with different lyophilized stock cultures:

Lyophilized stock culture	Growth of Step-2 seed culture (OD 660 nm)* 1(d)	2(d)	3(d)	4(d)	5(d)
Animal Milk (3-ABI-13)	0.51	0.81	0.96	0.43	0.33
Soy Milk (SM-409-0998-1)	0.59	0.90	0.88	0.43	0.38

*1 ml lyophilized stock culture into 10 ml of step-1 seed medium; 1 ml step-1 seed culture into 40 ml of step-2 seed medium in a 75 ml flask.

Table 50

Cell growth in fermentation medium

Lyophilized stock culture	Growth (OD 660 nm) 1(d)	2(d)	3(d)	4(d)	5(d)	6(d)	7(d)	9(d)
Animal Milk	0.37	0.62	0.79	0.78	0.49	0.34	0.30	0.27
Soy Milk	0.34	0.56	0.74	0.54	0.37	0.30	0.27	0.24

Table 51
Toxin Production

Stock	K_f		$L_f$toxin	
	7d	9d	7d	9d
Animal Milk	46	47	62.5	62.5
Soy Milk	49	49	52.5	57.5

Table 52

Effect of seed age on toxin production by a culture (SM-409-0998-1) derived from a soy milk stock.*

1st stage seed (h)	2nd stage seed (h)	Maximum growth (OD 660 nm)	Maximum Lf_{toxin}
24	24	0.69	62.5
48	24	0.68	37.5
48	48	0.65	42.5
*One expt.			

The results of experiments summarized in Tables 49-52 show that cultures of *C. tetani* lyophilized and stored in soy milk lacking any animal milk can be used to inoculate a seed medium for subsequent growth and production of Tetanus Toxin. In addition, Tables 49-52 show that production of Tetanus Toxin by *C. tetani* originating from cultures stored in soymilk and grown in soy seed medium and soy fermentation medium was comparable to levels of Tetanus Toxin production by *C. tetani* derived from cultures stored in animal milk. Table 52 shows the results of one experiment demonstrating that maximum toxin production occurs in a protocol using two seed growth stages when the culturing time for each stage is approximately 24 hours.

Example 18.

The Effects of Initial pH on Growth and Toxin Production.

The effects of initial pH on the growth of *C. tetani* and the production of Tetanus Toxin were examined. Table 53 shows that cell lysis (as measured by decreasing O.D.) varied according to the initial pH value of the medium. Generally, the degree of cell lysis decreased as the initial pH of the starting fermentation medium was increased. Table 54 shows that Tetanus Toxin production was highest when the initial pH of the fermentation medium was approximately 6.0-6.2.

Table 53

Cell growth in fermentation medium at different initial pH values.

pH	Growth (OD 660 nm)*							
	1(d)	2(d)	3(d)	4(d)	5(d)	6(d)	7(d)	9(d)
5.6	0.20	0.32	0.41	0.28	0.13	0.11	0.10	0.10
5.8	0.22	0.36	0.48	0.45	0.17	0.13	0.12	0.13
6.0	0.26	0.43	0.53	0.50	0.21	0.16	0.16	0.17
6.2	0.26	0.43	0.48	0.51	0.22	0.20	0.20	0.21
6.4	0.35	0.55	0.71	0.46	0.24	0.20	0.18	0.19
6.6	0.50	0.73	0.93	0.87	0.41	0.26	0.21	0.16
6.8**	0.37	0.62	0.79	0.78	0.49	0.34	0.30	0.27
7.0	0.37	0.61	0.78	0.71	0.41	0.31	0.28	0.26
7.2	0.70	1.01	1.13	0.99	0.75	0.44	0.35	0.39
7.4	0.37	0.75	0.88	0.88	0.65	0.45	0.41	0.41

*OD of second stage seed culture at the time of inoculation of fermentation medium: Animal milk lyophilized culture = 0.97.

**Control

Table 54
Toxin production with different initial pH of fermentation medium

pH	K_f		$L_f$toxin	
	7d	9d	7d	9d
5.6	49	46	57.5	62.5
5.8	40	43	62.5	62.5
6.0	40	41	67.5	67.5
6.2	40	41	67.5	67.5
6.4	42	50	57.5	57.5
6.6	63	51	52.5	47.5
6.8	46	47	62.5	62.5
7.0	57	43	57.5	52.5
7.2	103	61	27.5	32.5
7.4	83	54	32.5	32.5

Table 55
pH Measurements

pH	Before Autoclaving	After autoclaving	Final
5.6	5.6	6.1	7.4
5.8	5.8	6.2	7.5
6.0	6.0	6.4	7.7
6.2	6.2	6.5	7.8
6.4	6.4	6.6	7.7
6.6	6.6	6.8	7.3
6.8	6.8	6.9	7.6
7.0	7.0	7.0	7.9
7.2	7.2	7.2	7.9
7.4	7.4	7.8	8.1

Example 19

Production of Tetanus Toxin by *C. tetani* by fermentation in the presence of nitrogen or a nitrogen/hydrogen mixture

Table 56 summarizes data showing that high levels of toxin formation by *C. tetani* in fermentors receiving N_2 in the headspace continuously. When nitrogen was supplied for only the first day, toxin production was less than when nitrogen was supplied for 6-9 days. A black color arising in the fermentors with agitation was probably due to an oxidative interaction between the reduced iron powder, the microorganism and air leading into the fermentor. The black color is usually associated with poor production. When nitrogen was continually introduced for 6-9 days, black color did not appear and toxin production was good.

Table 56.

Toxin production in fermentors with continual N_2 introduced into headspace.

Vessel	Maximum Lf_{toxin}
In anaerobic chamber with 10% H_2 and 90% N_2 supply	
Test tubes	52.5
Bottles	47.5
With 100% N_2 supplied during the whole fermentation	
Fermentor #1	47.5
With 100% N_2 supplied during the fermentation for the first 6 days	
Fermentor #2	52.5
With 100% N_2 supplied during the first 24h	
Fermentor #3	32.5
*One expt.	

Table 57

Cell growth and toxin production in 1 liter fermentor with N_2 supplied.

F1 = Basic medium with 0/5 g/l iron powder (IP).
F2 = Basic medium + 0.2 g/l ferrous gluconate (FG)
F3 = Basic medium + 0.14 g/l ferric citrate (FC)
F4 = Basic medium + 0.16 g/l ferrous ammonium sulfate (FAS)

Media	1(d)	2 (d)	3 (d)	4 (d)	5 (d)	6 (d)	7 (d)	9 (d)
F1 (0.5 g/l IP)	0.42	0.82	0.92	0.90	0.85	0.85	0.86	1.20
F2 (0.2 g/l FG)	0.40	0.65	0.80	0.80	0.72	0.70	0.68	0.68
F3 (0.14 g/l FC)	0.41	0.64	0.78	0.78	0.72	0.70	0.70	0.70
F4 (0.16 g/l FAS)	0.40	0.66	0.76	0.75	0.70	0.69	0.68	0.64
	K_f							
Media	1 (d)	2 (d)	3 (d)	4 (d)	5 (d)	6 (d)	7 (d)	8 (d)	9 (d)
F1 (0.5 g/l IP)	no reaction	120	62	40	38	48	52	43	47
F2 (0.2 g/l FG)	no reaction	no reaction	100	76	63	60	62	55	53
F3 (0.14 g/l FC)	no reaction	no reaction	100	74	58	56	60	53	77
F4 (0.16 g/l FAS)	no reaction	no reaction	100	74	63	60	60	53	53
	$L_f$toxin								
Media	1 (d)	2 (d)	3 (d)	4 (d)	5(d)	6 (d)	7 (d)	8(d)	9 (d)
F1 (0.5 g/l IP)	no reaction	7.5	32.5	52.5	42.5	42.5	42.5	47.5	42.5
F2 (0.2 g/l FG)	no reaction	no reaction	7.5	22.5	27.5	37.5	32.5	37.5	32.5
F3 (0.14 g/l FC)	no reaction	no reaction	7.5	22.5	27.5	32.5	32.5	32.5	32.5
F4 (0.16 g/l FAS)	no reaction	no reaction	7.5	22.5	22.5	37.5	32.5	32.5	32.5

One experiment

Stock Culture of *C. tetani* used: *Clostridium tetani* SM-409-0998-1 (lyophilized culture of soy-milk, stored at 4°C).

Fermentation medium:

Basic medium	
Hy-Soy*	35g/l
Glucose	7.5 g/l
NaCl	5.0 g/l
Na_2HPO_4	0.5 g/l
KH_2PO_4	175 mg/l
$MgSO_47H_2O$	50 mg/l

An experiment summarized in Table 57 demonstrates that several iron sources are suitable for growth and toxin production. With agitation, optimal growth was observed with iron powder at approximately 2-3 days and optimal toxin production was observed at approximately day 4. Optimal growth and toxin production was observed with iron powder was used as the source of iron.

Claims

We claim:

1. A method for production of Tetanus Toxin, comprising steps of:

culturing an organism of genus *Clostridium* in a medium under conditions that allow production of Tetanus Toxin, wherein said medium is substantially free of animal-derived products.

2. The method of claim 1, wherein the step of culturing, the organism is *Clostridium tetani.*

3. The method of claim 1, wherein the step of culturing, the medium comprises at least one compound derived from a vegetable.

4. The method of claim 3, wherein the step of culturing, the vegetable is soybean.

5. The method of claim 3, wherein the step of culturing, the at least one compound derived from a vegetable is hydrolyzed soy.

6. The method of claim 5, wherein the step of culturing, the hydrolyzed soy is present in the medium at a concentration between approximately 10-300 g/L.

7. The method of claim 5, wherein the step of culturing, the hydrolyzed soy is present in the medium at a concentration between approximately 20-100 g/L.

8. The method of claim 1, wherein the step of culturing further comprises culturing *Clostridium* until cell lysis has occurred.

9. The method of claim 1, wherein the step of culturing, the medium comprises hydrolyzed soy, glucose, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, and iron.

10. The method of claim 9, wherein the iron is iron powder, iron wire, iron foil, ferrous gluconate, ferrous citrate or ferrous ammonium sulfate.

11. The method of claim 10, wherein the iron wire has a diameter of approximately 0.075 millimeters.

12. The method of claim 9, 10 or 11, wherein the medium further comprises activated charcoal.

13. The method of claim 1, wherein the step of culturing, the conditions that allow production of Tetanus Toxin comprise anaerobic conditions.

14. The method of claim 13, wherein the step of culturing, the anaerobic conditions comprise introducing nitrogen into a culturing chamber.

15. The method of claim 1, further comprising the step of recovering Tetanus Toxin.

16. A method for production of Tetanus Toxin, wherein said method comprises steps of:

culturing an organism of a genus *Clostridium* in a first medium under conditions that allow growth of said organism, wherein said medium is substantially free of animal-derived products;

inoculating a second medium with all or a portion of the first medium after the step of culturing, wherein said second medium is substantially free of animal-derived products;

culturing the organism in the second medium under conditions that allow production of Tetanus Toxin; and

recovering Tetanus Toxin from the second medium.

17. The method of claim 16, wherein the step of culturing an organism in a first medium comprises culturing *Clostridium tetani* in the first medium under conditions that allow growth of *Clostridium tetani*, wherein the medium is substantially free of animal-derived products.

18. The method of claim 16, wherein the steps of culturing an organism in a first medium and culturing the organism in a second medium, the first and second media comprise at least one compound derived from a vegetable.

19. The method of claim 18, wherein the compound is soy.

20. The method of claim 19, wherein the soy is hydrolyzed soy.

21. The method of claim 16, wherein the step of culturing an organism in a first medium, the medium comprises hydrolyzed soy, NaCl and glucose.

22. The method of claim 16, wherein the step of culturing the organism in a second medium, the second medium comprises hydrolyzed soy, glucose, NaCl, Na_2HPO_4, $MgSO_4 7H_2O$, KH_2PO_4, and iron.

23. The method of claim 22, wherein the second medium further comprises activated charcoal.

24. The method of claim 22, wherein the iron is selected from the group consisting of: powdered iron, iron wire, iron foil, ferrous citrate, ferrous gluconate, and ferrous ammonium sulfate.

25. The method of claim 16, wherein the steps of culturing comprise culturing under anaerobic conditions.

26. The method of claim 25, wherein the anaerobic conditions comprise introducing nitrogen gas.

27. The method of claim 16, wherein the step of culturing an organism in first medium, the medium is substantially free of animal-derived products and comprises hydrolyzed soy at a concentration between approximately 10-300 g/L.

28. The method of claim 27, wherein the step of culturing an organism in a first medium, the first medium is substantially free of animal-derived products and comprises hydrolyzed soy at a concentration of approximately 100 g/L.

29. The method of claim 16, wherein the step of culturing an organism in a second medium, the second medium is substantially free of animal-derived products and comprises hydrolyzed soy at a concentration between approximately 10-200 g/L.

30. The method of claim 29, wherein the step of culturing an organism in a second medium, the second medium is substantially free of animal-derived products and comprises hydrolyzed soy at a concentration between approximately 10-100 g/L.

31. The method of claim 16, wherein the step of culturing an organism in a second medium under conditions that allow production of Tetanus Toxin, the conditions comprise anaerobic conditions, agitation, nitrogen gas, and a culturing temperature between approximately 30-38 degrees Celsius.

32. The method of claim 16, wherein before the step of culturing, the method comprises the step of inoculating a first medium with a culture containing an organism of genus *Clostridium* stored in a culture medium substantially free of animal derived compounds.

33. The method of claim 16, wherein the step of inoculating a first medium, the culture medium further comprises hydrolyzed soy.

34. A composition comprising a medium substantially free of animal-derived products for culturing an organism and comprising an organism of the genus *Clostridium*.

35. The composition of claim 34, wherein the composition comprises at least one compound derived from a vegetable

36. The composition of claim 35, wherein the at least one compound derived from a vegetable is soy.

37. The composition of claim 36, wherein the soy is hydrolyzed soy.

38. The composition of claim 36, further comprising iron, glucose, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, and NaCl.

39. The composition of claim 37, wherein the hydrolyzed soy is at a concentration between approximately 10-200 g/L.

40. The composition of claim 39, wherein the concentration of hydrolyzed soy is between approximately 20-100 g/L.

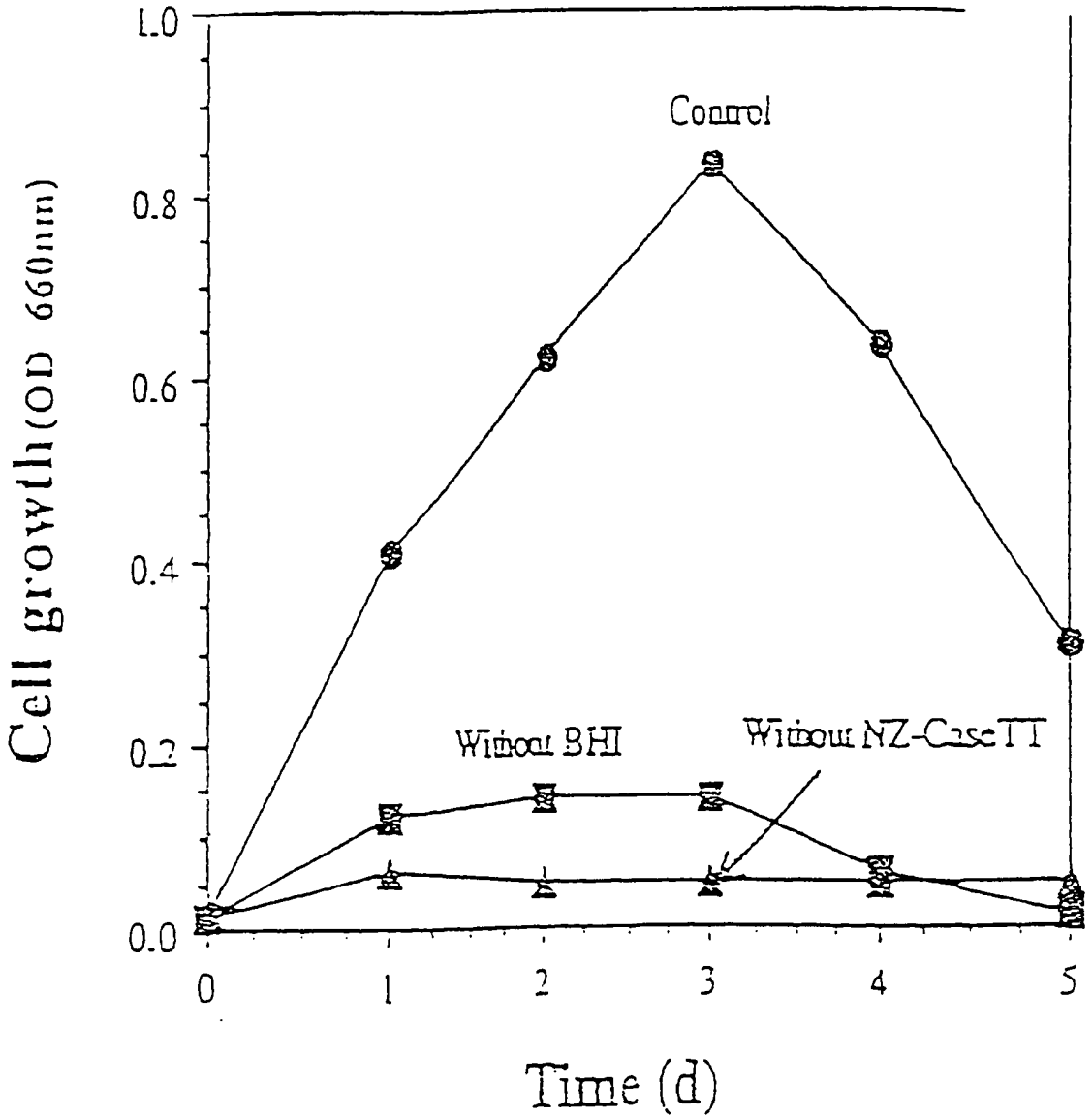

Cell growth (OD 660nm)
1.0
0.8
0.6
0.4
0.2
0.0
Control
Without BHI
Without NZ-Case TT
0
1
2
3
4
5
Time (d)

FIGURE -a-

Fig.2. The activity of soluble soy products as BHI replacements for cell growth.

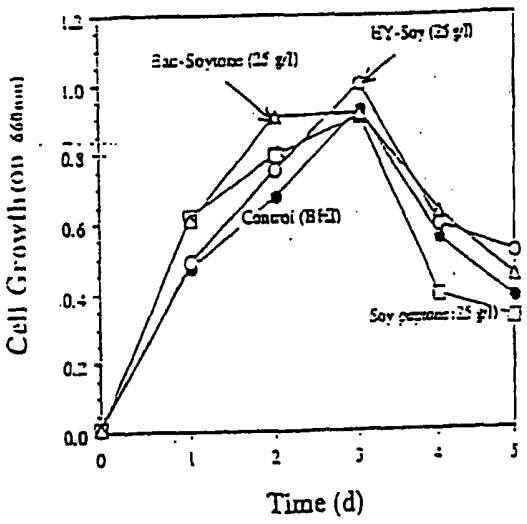


FIGURE 2

Fig. 3. The activity of insoluble soy products as BHI replacements for cell growth.

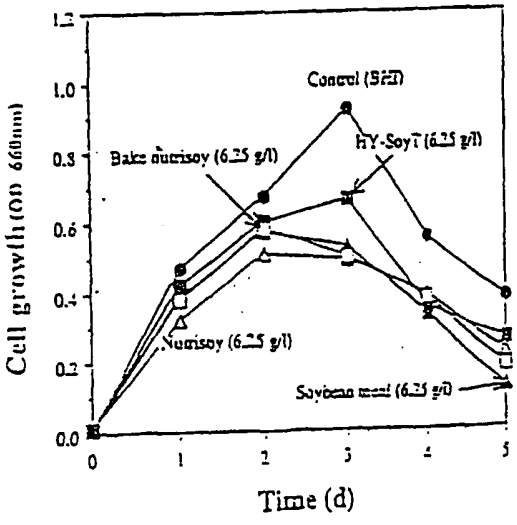


FIGURE 3

Fig. 4. The activity of different soy products (from Quest) as BHI replacements for cell growth.

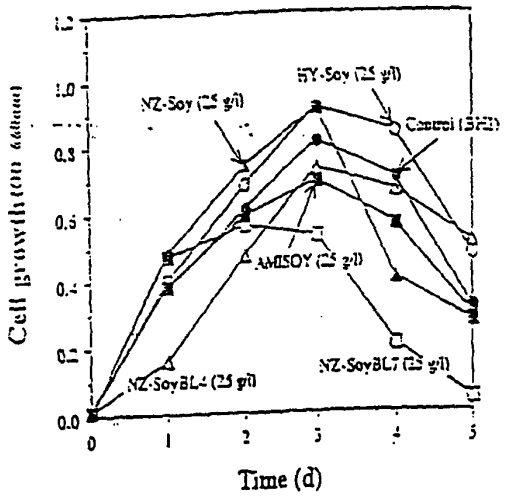


FIGURE 4

Fig. 5. The activity of different soy products as BHI replacements for cell growth.

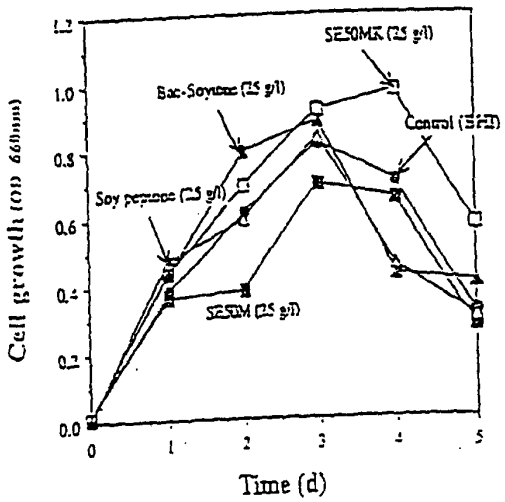


FIGURE 5

Fig. 6. Comparison of cell growth with 2 concentrations of BHI and Quest HY-Sov.

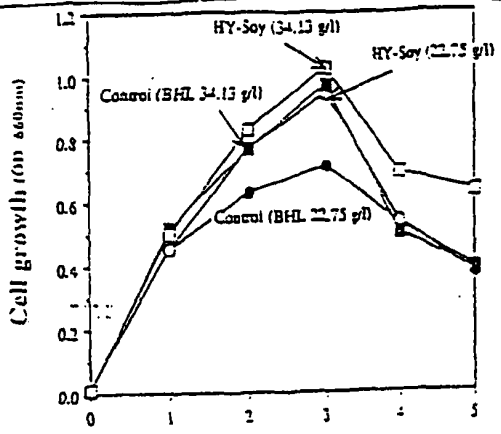


Fig. 7. The ability of different Quest soy products (22.75 g/l) to replace BHI for cell growth.

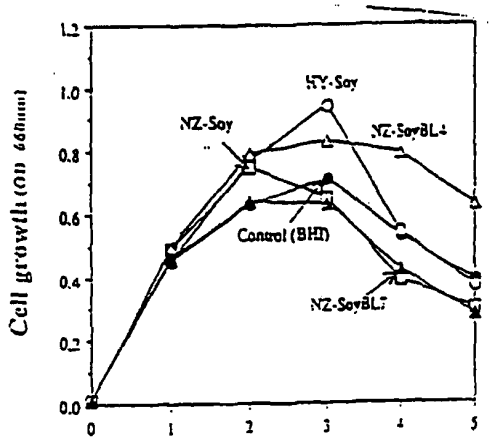


FIGURE 7

Fig. 8. The ability of soy products (22.75 g/l) to replace BHI for cell growth.

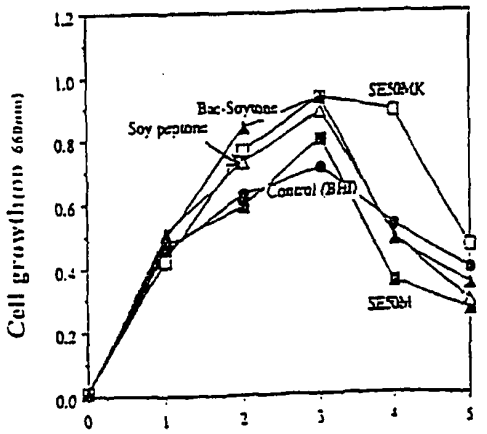


FIGURE 8

Fig. 9. The effect of removing NZ-CaseTT on cell growth in media with BHI vs. soy peptones.

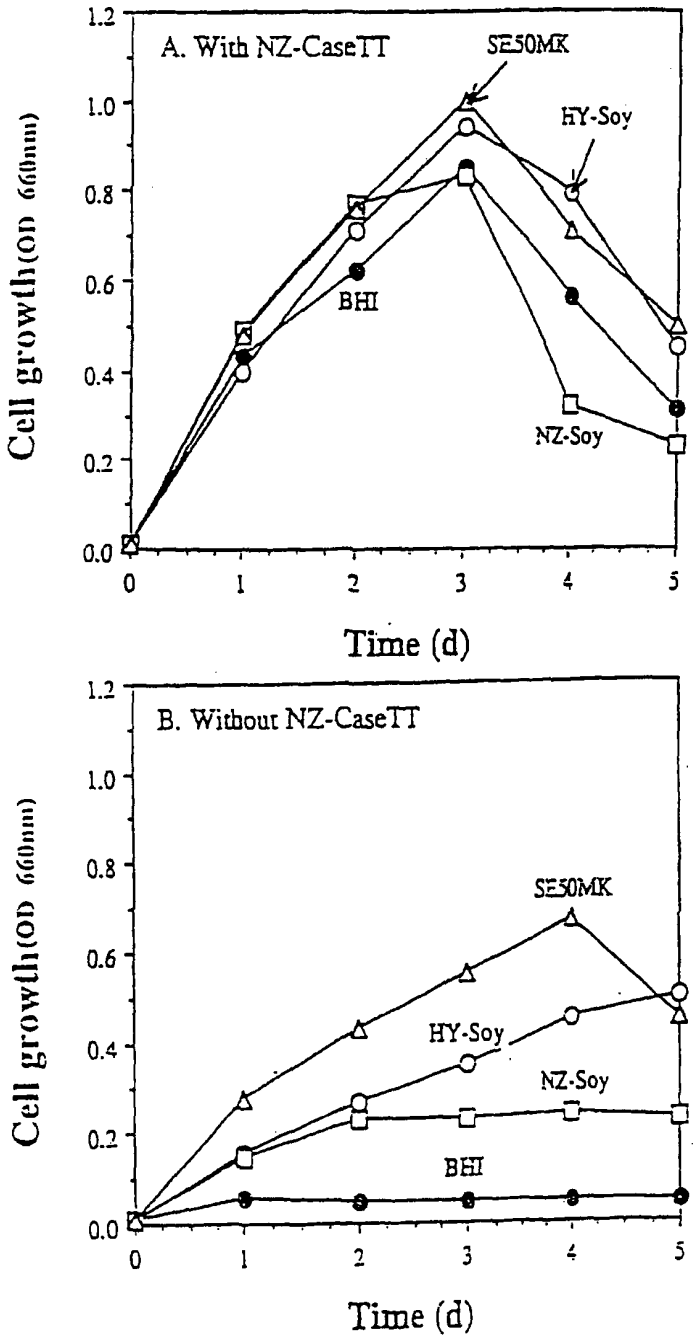


FIGURE 9

Fig. 10. Partial replacement of NZ-CaseTT by yeast extract for growth.

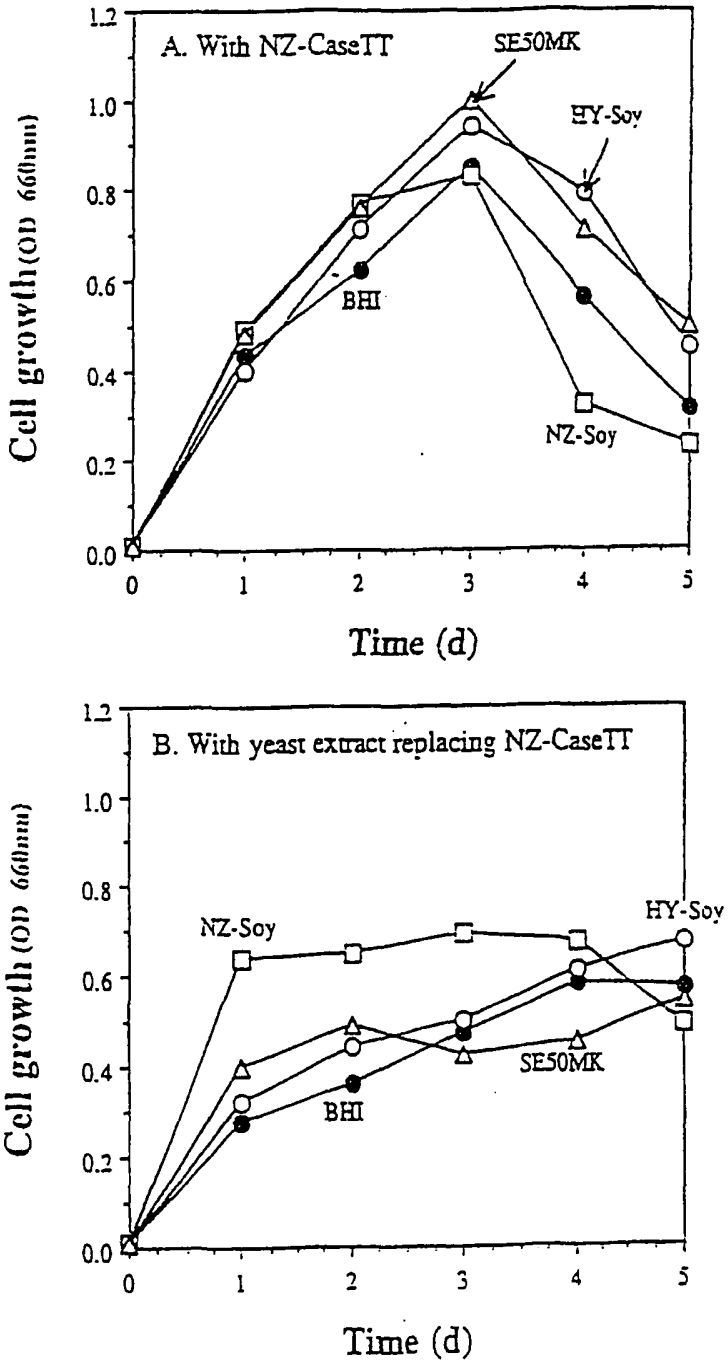


Figure 10

FIGURE 11

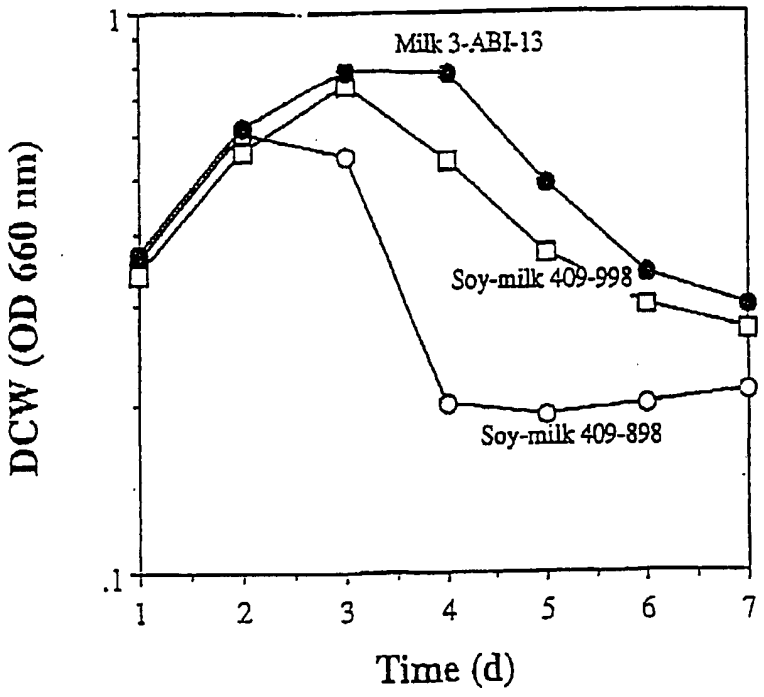

1
.1
DCW (OD 660 nm)
Milk 3-ABI-13
Soy-milk 409-998
Soy-milk 409-898
1
2
3
4
5
6
7
Time (d)

Fig. 12. Comparative effect of yeast extracts and malt extracts as replacements for NZ-CaseTT in the presence of HY-Soy.